RECURRING MICROBIAL MUTATIONS, AND THEIR ASSOCIATION WITH EPIDEMIC CYCLES AND EVOLUTION

THESIS

submitted for the degree of

DOCTOR OF MEDICINE

of the

UNIVERSITY OF EDINBURGH

by

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October, 1964.

THE UNIVERSITY *of* EDINBURGH

"Bacteria must be studied, not only in the effects which they have on practical human problems, but, also for what they do as independant living organisms."

R. Dubos, 1949

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SUMMARY

This is an account of a 12-year search by bacteriological methods to explain the cyles of the great infectious fevers.

In a historical survey we see that epidemics have shown long trends over the centuries and often shorter regular waves, such as that every 2 years in measles, as well. Exclusion of seasonal and other extraneous factors suggests a rhythm in the microbe itself.

In Part II experiments with Escherichia coli show not only that streptomycin-resistant mutations are mathematically predictable for each type-strain, but that these variants mutate in turn at regular rates, while "back-mutations" also occur. Similar predictable and progressive mutations could explain the sudden appearance and reappearance of new characteristics such as exalted virulence or infectivity.

In Part III the origin and development of similar streptomycin-insensitivity is considered in Mycobacterium tuberculosis. Using a new method, Slope Diffusion, we see the emergence of mutants over a number of years in 25 cases and in 5 families or groups, culminating in what is virtually a new disease - streptomycin-resistant tuberculosis.

There is a constant mobilis in mobile between parasite and host as each adapts to changes in the other. In Part IV it is suggested that the pre-determined frequency of microbial mutations explain epidemic cycles, and that evolution is no more fortuitious than the more successful of these fixed mutations on which it depends.

PART I.

CYCLIC FLUCTUATIONS IN EPIDEMIC DISEASES

CHAPTER 1. THE HISTORICAL INCIDENCE OF EPIDEMICS.

INTRODUCTION

Although it would seem that the great infectious diseases have always been with us it is axiomatic that their incidence has been by no means uniform down the centuries. History is made up of epidemic epochs. Plague devastated Mediaeval Europe, smallpox pock-marked all Christendom in the Eighteenth Century, and anginous scarlet fever and diphtheria exterminated entire Victorian families. In our own day worldwide pandemics of influenza remind us of the scourges of the past.

Quite a superficial study soon shows that these long-term trends are themselves fluctuating with a constancy which is so remarkable as to seem at first inexplicable. An annual rise and fall could, of course, be seasonal or due to other climatic or even social factors; but how are we to account for the waves of measles every second year, or of influenza pandemics returning twice or thrice in a century? The aim of this thesis is to survey both these long trends and the intervening recurring cycles, and to show that their regular rhythm is not only explicable; but must in fact be characteristic of mutations, which alone could enable a living microbe to survive by constantly adapting to the changes in its host.

We will start by surveying the historical aspects of epidemic cycles, culminating in a study of influenza, whose vagaries are almost certainly mutational and afford the best evidence for that purposeful adaptation described as "epidemiological drift".

EPIDEMIC CYCLES AND THEIR IMPACT ON HUMAN HISTORY

It is often supposed that the unprecedented advances in medicine of the present century are due to the same rise in technology which has revolutionised other branches of science. No organ remains inaccessible to modern surgery, and the pathogenic bacteria, only discovered in the 1880's and since, have for the most part been miraculously conquered by the antibiotics of the last twenty years. But whether preventive medicine has appreciably influenced the vagaries of epidemic disease is much more in doubt.

Nowhere is this better seen than with plague. The World Health Organisation recently surveyed the whole globe except China and found only 300 cases for 1959, [479] and yet in historical times four pandemics of plague ravaged the known world. The first in Trajan's reign affected his whole empire for half a century, the "Black Death" of the 14 th. Century killed perhaps 40 million - half the world.

The "Great Plague" of the 17 th. Century which swept London in 1665 was commemorated by Pepys, and the 1894 pandemic killed 12 million in India alone in the subsequent 43 years [298].

In Shakespeare's day the plague visited London every summer and when the deaths rose to thirty a week the theatres were shut. But in "Timon of Athens" [392] published in 1623, Shakespeare makes Timandra voice a common belief that disease transmitted to another left the infecter free: "I will not kiss thee; then the rot returns to thine own lips again". So that it is hardly surprising that the lues was rampant.

Chaucer [66] says of the plague in his Pardoner's Tale: "He hath a thousand slain this pestilence," and he goes on to describe the prevalence of smallpox - 'the pokkes', and 'mesel' or leprosy. Food-poisoning epidemics, commonplace in the Middle Ages, covered the machinations of the Borgia poisoners, [184], and syphilis, "the strange disease" traditionally brought by Columbus' men from the Hispaniola [398], followed Charles the Eighth's army across Europe. Cromwell died of malaris [68] then epidemic every autumn [336] in the marshes of Pall Mall, and plague, smallpox and dysentery can be identified among the fevers which made up two-thirds of Sydenham's practice [78].

Although measures for the control of leprosy and other "unclean issues" had been given in the Mosaic law [266], including isolation of cases and contacts and washing or burning of their clothes, no such enlightened prevention prevailed in this country for many centuries. Thus Chaucer [66] in the fourteenth century wrote of the 'docteur': "He kepte that he wan in pestilence," and Bullein in his "Dialogue against the Pestilence" wrote in 1573: "And in such plagues we poor people have mickle good ... Besides us pakers, many more men have good luck, as the vicar, parish clerk and the bell-man; we look for old cast coats, jackets, hose, caps, batts and shoes, by their deaths which in their lives they would not depart from, and this is our hap" [469].

Smallpox, unheard of before the time of James I, killed 50,000 of the half million population of London in the last 40 years of the 17 th. Century, and exterminated tropical populations when it had waned in Europe. Cholera appeared in England in 1817 and disappeared in 1892 regardless of unchanged sanitation.

Scarlet Fever was notable only for a rash from its first description in the 16 th. Century to Sydenham's account in the 17 th.[78]. In the 18th. it raged as a fever all over Europe, diminishing in the early 19 th. Century only to become highly virulent again from 1830 to 1870. Since 1870

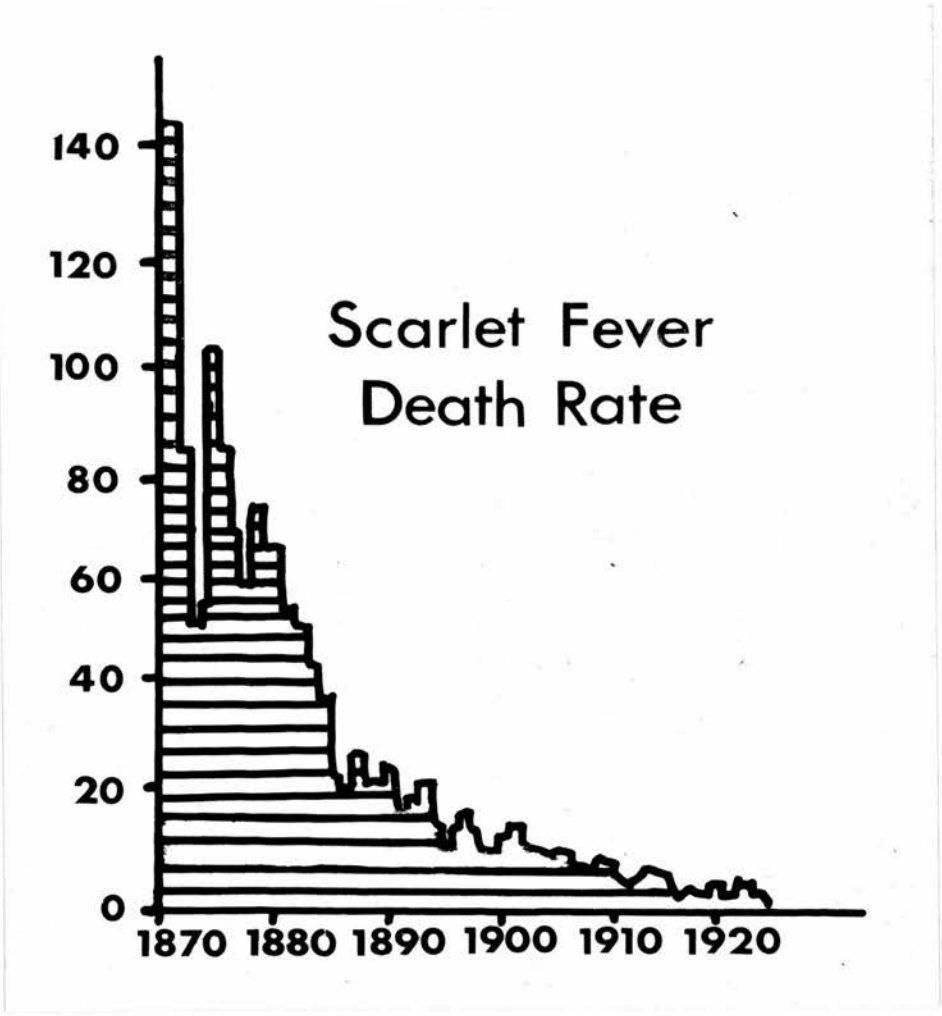


Fig: 1. *Deaths from Scarlet Fever had fallen strikingly from 144.6 per 100,000 of the population of England and Wales in 1870 to 1.2 in 1936* [147], *as shown by this diagram after Madsen* [291]. *This decline, due almost entirely to lowered fatality, and hardly at all to any falling incidence, preceded the introduction of sulphonamides and penicillin.*

the death rate of 720 per million fell to 11 per million in the 1930's, too soon to be influenced by chemotherapeutic drugs (Fig: 1). Moreover the other manifestations of virulent haemolytic streptococci - erysipelas and puerperal fever - fell too with remarkable consistency. Clearly the long-term fluctuations in the incidence of these, and the other infectious diseases cited in this thesis, present an enigma which has eluded men's understanding for centuries.

THE INFLUENCE OF OVERCROWDING

In Shakespeare's "Henry IV" we hear: "As men take diseases one of another; let men take heed of their company," and again: "We are all diseased, and with our surfeiting and wanton hours have brought ourselves into a burning fever" [390]. Sir Thomas Browne observed to a friend, half a century later: "The small pox grows more pernicious than the great: the king's purse knows that the king's evil grows more common. Quartan agues are become no strangers to Ireland; more common and mortal in England," [466] and Defoe [91] wrote resignedly in 1720: "We were crowded enough to cause a pestilence among us." Nowhere was overcrowding worse than among children, generally the most susceptible of all to infectious diseases, - "and in the morn and liquid dew of youth contagious blastments are most imminent," as we read in "Hamlet" [391].

Boarding schools offer the opportunity for studying the fluctuations of diseases in more or less closed herds of non-immunes, and when one recollects that the English and Scottish Public Schools have had unbroken histories extending back, in some cases, over a period of five centuries, even their fragmentary medical records assume importance. The diet did little to maintain the boys' resistance, and both Coleridge [75] and Charles Lamb [249] have written of the hunger of their schooldays at Christ's Hospital, while as late as 1846 the boys at Winchester rose at 5.30 a.m. and waited 4½ hours for breakfast of bread and beer, of which the nurse at the sick house disposed of three quarts a day.

Plague killed 26 boys and 11 masters at Winchester in 1430, the dying boys being immediately replaced by others. As a preventive Eton obliged all its boys to smoke in school each morning under penalty of a whipping, and in 1450 issued a paper asking whether a new boy had been exposed to the Great Death. But the younger boys were still sleeping on the floor of Long Chamber there a hundred years ago with the rats eating their clothing and braces, while at Westminster single beds cost £4 extra, a dubious luxury when fagging began at 3.30 a.m. [263].

During the 19 th. Century Christ's Hospital had only one towel for every four boys, and ringworm was treated by the boys themselves with ink. Children with fevers

traditionally received emetics and purges, and had their temperatures lowered by bleeding and cold showers.

While admitting that more humane boarding schools have reduced epidemics by increasing the boys' stamina it seems certain that cyclic changes in virulence may have also occurred, and Lempriere [263] has shown that school records reveal four periods of different predominant infectious fevers. Thus 1400 - 1700 was a period of plague which usually led to rustication or closure of the school, while from 1700 - 1800 small-pox covered Europe. 1800 - 1870 was the hey-day of diphtheria and the exanthemata, a time when anginous scarlet fever and diphtheria were not well distinguished and equally fatal, while from 1870 influenza caused the major epidemics in schools as elsewhere.

SPECULATIONS ON THE CAUSE OF EPIDEMICS

History asserts that Sennacherib lost 165,000 men in one night on the eve of attacking Palestine in 711 B.C., and the gratified Jews claimed divine visitation. Hippocrates, three centuries later, described the reigning diseases through successive seasons of the island of Thasos, associating their onset with the changes of the weather: "About the equinox, and until the season of the Pleiades, and at the approach of winter, many ardent fevers set in" [195]. The advent of Syphilis in Europe was ascribed at tne time to the conjunction

of the planets Venus, Mars and Mercury in 1483 [398], and about 1600 Galileo's telescope provided the credulous with many astronomical phenomena suitable for appostion to the recurrences of disease. Similarly the microscope with its revelations of the microparasites of man, swept all medicine before it in the '80's, so much so that it was in distinctly bad taste to admit any other causative factor than Pasteur's Germ Theory of Disease [184].

Although the early microscopists overstated their case to the point of ridicule, the full potentialities of bacteria and viruses have still to be revealed and Pasteur may yet be vindicated. To a biologist all living things are the products of evolution, and to a microbiologist, accustomed to the prodigious multiplication of bacteria, mutations are a daily observation. But even he may scarcely realise the full significance of such changes among organisms which, in a single day, can complete as many generations as the human race has achieved in the whole Christian era.

The doctrine of the "Epidemic Constitution" was originated by Hippocrates, who, after describing the weather, argued that each season had a ruling disease [196]. Sydenham followed his classification remarkably closely and gave the Great Plague of London the same place in his series of years as the Fourth Constitution of Hippocrates, which was the

Plague of Athens as described by Thucydides. Creighton [80] remarked that fevers proper to the climate of Thasos were not likely to be identified in or near London except by a forced construction, and Sydenham appears to have carried back his records solely to accommodate the intention in view. Similarly Hamer [184] attempted to draw an exact analogy between epidemics in London between 1888 and 1928, and those of Sydenham two hundred years before. He traced influenza to the Milaires and Picardy Sweats of earlier centuries, and likened its protean tendencies to Sydenham's "exceedingly anamalous and irregular fever". He suggested comparison of the epidemic wave of measles with the far less stable influenza, and regarded typical influenza as the "spray on the crest" of other fever waves, instancing pneumonia in the '90's and meningitis in 1918. In short the Epidemic Constitutionalists insist that there are periodic recurrences down the centuries of like epidemic constitutions.

In 1918 the concurrence and resemblance of cerebrospinal fever and poliomyelitis on the one hand, and of influenza, bronchitis and pneumonia on the other, encouraged some official recognition of the theory [327], and Hamer asserted that all these diseases were but protean manifestations of the one epidemic [183]. But McNalty [289] drew attention to the resemblance of the prodromata and abortive cases of poliomyelitis and encephalitis lethargica

to the ubiquitous influenza, and Hall [181, 182] ironically remarked that, if there was any connection between the diseases, it was unusually well concealed from the clinician.

The alternative theory of the "Epidemic Cycle" had been postulated by Whitelegge [463] in 1892, who had considered that minor epidemics of measles became progressively intensified into the big cycles of major ones, and Hirsch gave similar explanations for influenza, and the less invasive cerebrospinal fever, noting that an increase in sporadic cases preceded and followed the main epidemics [289].

In 1928 Gill [159] propounded the "Quantum Theory of Epidemics" which purported to explain all epidemic phenomena in terms of a change in the relationship between the infection and immunity 'quanta', and in 1935 Topley and Greenwood [174] interpreted their experimental findings in mice in terms of an immunological constitution of the herd in relation to all the parasites to which it was exposed. By intermittent introduction of fresh mice into a herd heavily infected with rodent typhoid, these workers were able to propagate waves of fatality by low immigration where high immigration gave a steady death rate; but they admitted that virulence and infectivity varied independently even in the course of a single long epidemic, and the results do little to explain fluctuations in incidence.

The introduction of antibiotics provides a unique tool for observing changes in the bacterial organism, for resistance to these drugs is immediately conspicuous, and usually follows a genetic distribution. The resulting mutations make a fascinating study which may throw some light on epidemic cycles, and perhaps on the origin of sporadic cases of disease. Moreover the possibility of a new menace from epidemics of drug-resistant organisms cannot be dismissed.

Preliminary experiments suggested that various drug-fast mutations were in fact constant for any species, and, therefore, predictable, and in this thesis are described a few experiments measuring the mutation rates. It soon became clear that the number and diversity of mutations occurring simultaneously are so vast that this could be the challenge for almost any new circumstance, and go far to account for the onset of disease. Before describing these laboratory experiments, however, I would like to survey the astonishing fluctuations in the prevalence of infections to show the extent of the problem which they pose.

Quite a cursory study will show that all the common infectious diseases show one or more different types of fluctuation, be it periodic cycle, seasonal prevalence, irregular epidemicity or long-term trend [147]. The periodic

cycle, so well seen in the biennial incidence of measles and triennial of whooping cough [414], may be explained in part at least by the accumulation of a susceptible population exposed to sudden risk from cases or carriers - "and by the hazard of the spotted die let the spotted die" as Shakespeare expresses it [392]. The seasonal incidence appears to derive in part directly from climatic exposure and in part indirectly from the resulting enforced overcrowding or diminished ventilation or an infection due to these factors may predispose to another. Moreover abnormal weather may account for some irregular prevalences, for instance the heat wave of 1921 was associated with a high incidence of diarrhoea but a low rate for scarlet fever [147].

Schools isolate 16% of the population in term-time, at least for the greater part of the day, and school holidays materially affect the spread of disease between different places and different age-groups, and account for the timing of some epidemics. Similarly the British Isles are unusually well insulated against diseases from abroad - "this fortress built by Nature for herself against infection and the hand of war" [389] - so that the introduction of exotic infections is associated with a short meteoric career.

As regards long-term trends all the common infectious diseases have declined in virulence in recent years, but the

most providential deliverances in historical times have been from the "black" and "White" plagues, P. pestis and M. tuberculosis. The Great Plague or Black Death of the 14 th. Century was by far the most frightful epidemic in history. Beginning in China in 1346 it killed 13 million in less than a year and then spread to Europe along the trade routes depopulating the countries through which it passed, and even in England and Norway, where the epidemic ended, more than half the people were destroyed. Pope Clement VI ordered a census in 1348, and awe-struck messengers whispered that half the known world was dead, and offered a final estimate of 42,836,486, which was probably exceeded. Three hundred years later the second pandemic described by Pepys and Defoe killed one-third of London before the Great Fire of 1666 stopped its spread.

Tuberculosis, even if it has not vanished like the Plague, has markedly diminished in severity in this country although the down-trend faltered during the war [106]. Phthisis is now increasing in severity in India [8] and the Far East [396] generally just as it increased in this country in former centuries. Browne [466] in 1690 noted how often unsuspected tuberculosis was found at post-mortem examination, and everywhere its ravages were watched with the same superstitious dread [371] shown by the natives of Samoa and the New Hebrides today. There seems little doubt, in fact, that

we in Europe are meeting tuberculosis on the down-curve of an exceedingly long epidemic cycle, a cycle whose wave-length must be measured in centuries. The present decline began before the introduction of sanatoria and other preventive measures [53], just as the fall in diphtheria fatalities in 1890 preceded the introduction of antitoxin, that of scarlet fever in 1870 the general introduction of fever hospitals, and erysipelas and puerperal fever the discovery of the 'sulpha-' drugs.

How long these down-trends will continue none can at present say, but Topley and Greenwood [174] maintain on experimental grounds that a disease will never normally die out in a large community, and that, as active immunity merely prevents overt disease not infection, even mass immunisation would not eliminate a disease.

Centuries of co-existence have produced a balance between living species which, in the case of man and his parasites, must imply a constant mobilis in mobile phenomenon between the two. Mutations in the parasite gross enough to overwhelm the host will produce disease, and among communities an epidemic can follow and persist until some compensation restores the former equilibrium. Thus a distinction between commensal and pathogenic flora is really quite untenable for any parasitic micro-organism has in its protoplasm the key to virulence, and dismissal of, for example, coagulase-negative Staphylococci in a blood culture as harmless contaminants could be a quite unjustified assumption [85, 363].

CHAPTER 2. CLIMATIC AND OTHER RECURRING EXTERNAL FACTORS.

SEASONAL INCIDENCE

Variations in disease incidence according to the time of year have also attracted comment throughout history, although the fatalistic "to everything there is a season" [112] discouraged the search for a cause. Hippocrates [195] repeatedly noted the greater frequency of dysentery during the summer and autumn on Thasos, and the curious predominance of fevers around the equinoxes, still seen to this day. Thus while the majority of epidemic diseases in Europe are at their height in the late winter and spring, scarlet fever and poliomyelitis are autumnal in incidence, while typhus usually occurs in midwinter and only the intestinal infections reach their maximum prevalence during the summer. It seems possible that sudden changes in climate are more weakening to both humans and animal vectors than the relatively prolonged exposure to cold and wet in midwinter; but one cannot ignore social rhythms such as indoor and outdoor life, and the opening and closing of schools [174].

In the tropics the influence of climatic changes on epidemics is, as one might expect, still more marked, and to illustrate this malaria and dysentery incidence were surveyed in the laboratory records of an Indian garrison town over the

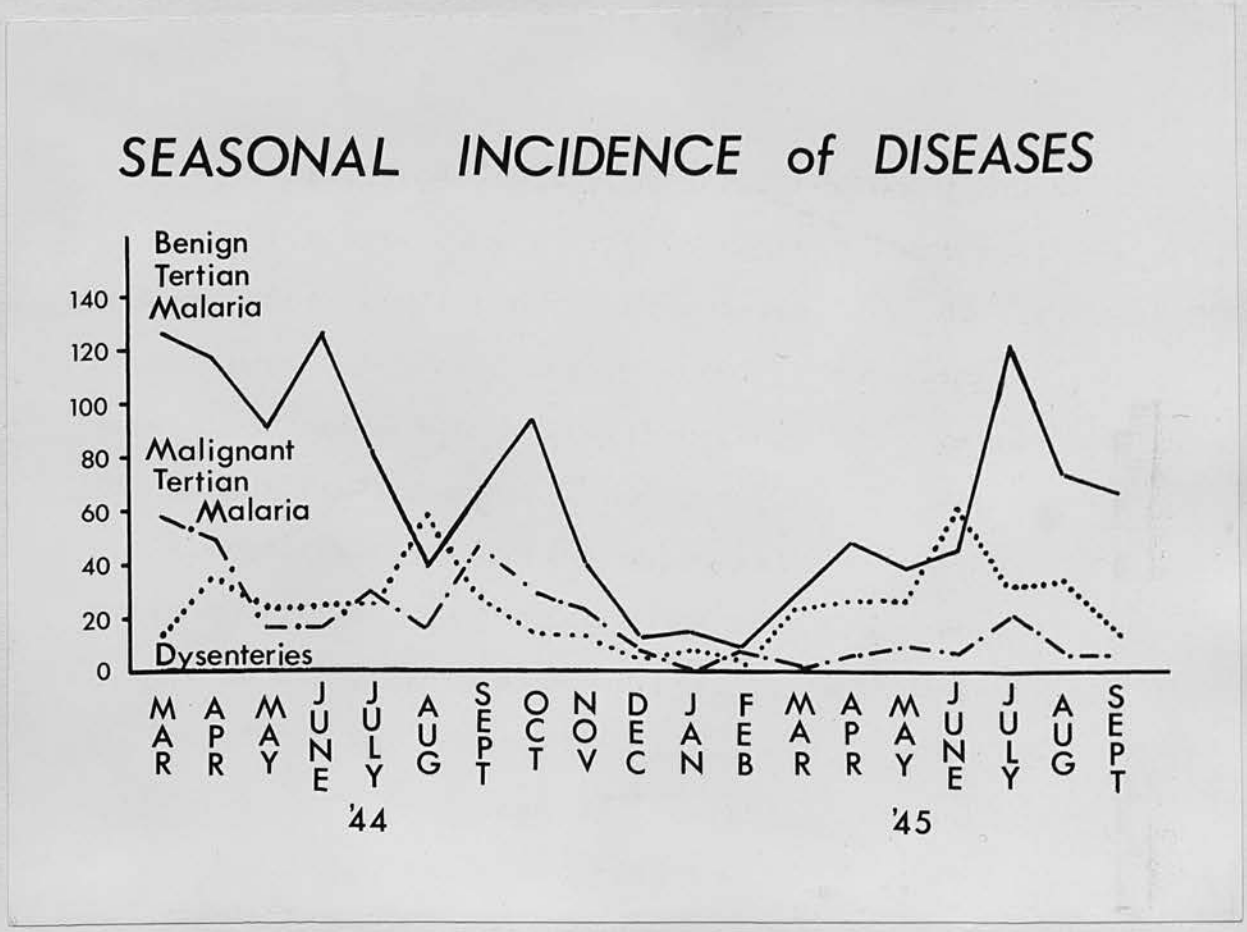


Fig: 2. *Incidence of diseases among British troops in Ahmadnagar, India, during the 1939 - 1945 War (Author's figures). After high initial incidence due to the arrival of infected troops in the spring of 1944, there were abrupt rises in malaria, and to a lesser extent in bacillary and amoebic dysenteries, each summer with the coming of the monsoon.*

two year period 1943 - 1945. It will be seen that malaria after an initial wave due to newly arrived infected troops, reappears abruptly each summer with the coming of the monsoons bringing breeding of its anopheline vector. Intestinal infections show less variation, but again there is a midsummer peak coinciding with increased flies (Fig: 2).

SIMULTANEOUS EPIDEMICS

There is no doubt too that one epidemic predisposes to another, and as early as 1847 Farr noted the co-existence of influenza, whooping-cough, measles and typhus all highly prevalent together, and in 1887 Wellington School had coincident scarlet fever and diphtheria, with influenza [263]. Scarlet fever is commonly associated with anterior nasal diphtheria, chickenpox, erysipelas and erythema nodosum [223], diphtheria also occurs with measles [129], measles with whooping-cough [236], C.S.F. with shingles, syphilis with gonorrhoea, typhus with relapsing fever, and cholera with influenza [47]. Both poliomyelitis and cerebro-spinal fever preceded the influenza pandemics of 1890 and 1918 [289], and a secondary broncho-pneumonia usually complicates measles while herpes labialis is a well-known concomitant of other infectious diseases. As Greenwood and Topley remark: "The association of two bacteria or, more especially a bacterium with a virus, may play a part in an epidemic no less than that played by variations of a single parasite" [174].

TABLE 1

FREQUENCY OF ORGANISMS ON FIRST ISOLATION IN SIGNIFICANT BACTERIURIA IN CASES WITH NO ABNORMALITY OF THE URINARY TRACT.

(personal communication from J. McGeachie, Glasgow Royal Infirmary)

E. coli	150
P. mirabilis	3
Citrobacter freundii	2
P. aeruginosa	1
Staph. albus	2
Strept. faecalis	0
E. coli and Strept. faecalis	2
Other mixed infections	0
	160

On the other hand one can but wonder at the purity of so many diseases. For instance several observers [156, 293, 358, 364, 383, 387] have noticed that mixed urinary infections are less common than simple ones; but the implications seem to have been overlooked, or a vain search made for some antagonism [387]. Even in chronic cases of stasis from prostatititis [156, 358], paraplegia [387], or cystitis [293], where a second pathogen could more readily join the primary invader, cultures more often than not yield a pure growth of one organism, usually a coliform (Table 1). If mere lack of hygiene and lowered resistance were alone responsible for pyuria one would expect to find as a rule the full range of intestinal flora represented. Presumably a mutation giving enhanced virulence to one organism at the opportune moment plays a far bigger part than is generally suspected.

Where no mutation is involved, as must generally be the case among the larger invertebrate parasites, whose reproduction rate is relatively slow, infection must just reflect the local fauna. Thus where, as in India, helminth parasites are plentiful cases of multiple infection are by no means uncommon (Table 2).

Infants might be supposed to offer little resistance to invading bacteria; but in fact even the youngest child

TABLE 2

SINGLE AND MULTIPLE HELMINTH INFESTATIONS IN AN INDIAN TOWN. AHMADNAGAR, SEPTEMBER 1945

Strongyloides stercoralis	3
Ascaris lumbricoides	31
Ancylostoma duodenale	37
Trichuris trichura	12
Heterodera marioni	1
Taenia saginata	3
Ancylostoma, Trichura & Ascaris	1
Ascaris & Taenia	1
Ascaris & Strongyloides	2
Ascaris & Ancylostoma	7
Ascaris & Trichuris	3
Ancylostoma & Trichuris	1
	102

presents a barrier demanding exalted virulence. This was illustrated by investigating infantile gastro-enteritis due to E. coli neapolitana in Edinburgh during six months (January to July) 1952.

During this period 167 babies were admitted to the Royal Hospital for Sick Children and the City Hospital for enteritis. Slide agglutinations were performed on a dozen E. colo colonies selected at random from each child's stool. As shown in Table 3, 37 had initially pure cultures of the Aberdeen α (O111.B4) serotype, 39 Aberdeen β (O55.B.5), while only 1 had both. Both types of infection were treated in the same wards relying on barrier nursing, confidence in which was generally justified by 3 healthy babies remaining unaffected by a month's stay in the ward, including the twin sister of a girl with the β strain.

Sulphadiazine virtually sterilised the faeces, and on stopping treatment E. coli communis reasserted its predominance, occasionally preceded for a few days by Proteus species, Enterococci or E. coli aerogenes. It is interesting to speculate why the normal flora can be relied upon to restore itself rather than a recurrence of the pathogens after their transient elimination. Four 'relapses' did indeed happen; but as a repetition of the agglutination tests showed a change of serotype in three instances, complete reinfection must have occurred in these cases.

TABLE 3

INCIDENCE OF E. COLI SEROTYPES IN CASES OF INFANTILE GASTRO-ENTERITIS AT R.H.S.C. & CITY HOSPITAL, EDINBURGH, JAN. - JULY 1952.

	'Aberdeen a'	'Aberdeen β'	a + β	Nether
Uneventful recovery	35	37	1	90
a → β	1			
β → a		2		
a → a	1			
Totals	37	39	1	90

a = Serotype 0111.B4

β = Serotype 055.B5

Presumably the pathogenic serotypes of E. coli neapolitana must be of low prevalence in humans until a mutation offers exalted virulence. This could explain, too, the replacement in Britain since 1952 of the 0111.B.4, and 055.B5 strains by 026 and 0119.[256]. All these strains are said to persist in farm animals [361, 362] and pets [309]. Given such large reservoirs virulent mutants can constantly menace babies, and that E. coli neapolitana has high mutation rates is shown in Chapter 7.

CHAPTER 3. NOVEL AND CHANGING CHARACTERS IN HUMAN EPIDEMICS

Butler [54] pointed out that an epidemic might be a great increase in a normally endemic disease, or a relatively much smaller prevalence of an exotic disease scarcely achieving the endemic heights of the other; but quite as noticeable because of its usual rarity. Among these more ephemeral infections are epizootics of animals, whose occasional epidemics in man could be explained by a mutation in the parasite enabling it to change its host [325].

INFECTIOUS DISEASES TRANSFERRING FROM ANIMALS TO MAN

Undulant Fever

Abortus fever is common enough in cows [427] to affect 5% of British milk samples and 10% of American; but up to 1929 only 14 known human cases had occurred in England and 350 in the United States [11, 36, 58, 65, 187, 428, 438, 477]. Even allowing for fallacies due to improved diagnosis [26], sporadic and epidemic cases in man do seem to have become increasingly frequent [86].

Eyre [122] believed that the corresponding melitensis septicaemia was primarily a disease of goats in the Persian hills and accompanied them in their world-wide wanderings,

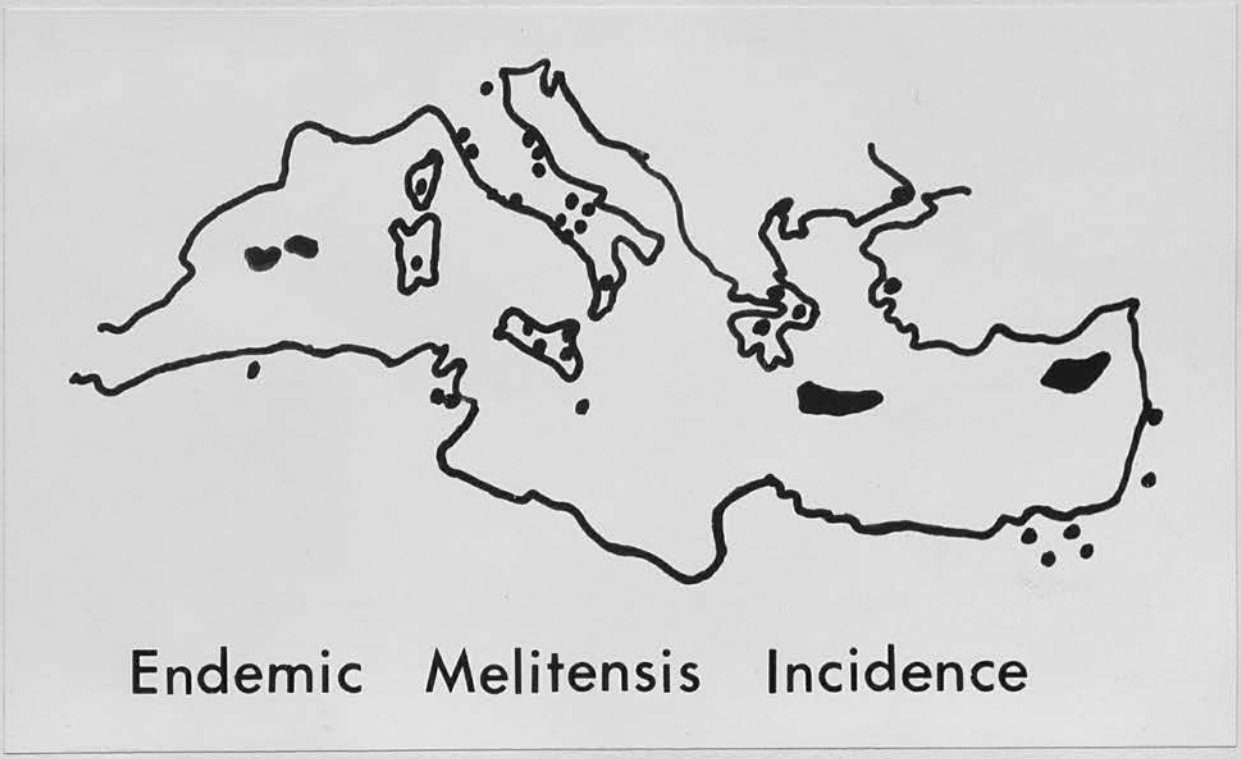


Fig: 3. *A geographical spread. Malta Fever, which affected 40% of the goats on that island in 1904 had spread round the Mediterranean by 1910; but the human cases were still limited to the coastline (after Eyre [122]).*

becoming modified for cows and other animals. A large epidemic of "Mediterranean or Gastric Remittent Fever" [206] occurred in 1856 in British troops returning from the Crimea, and the continued prevalence in Malta led to the discovery in 1904 that 40% of the goats there were healthy carriers, few herds being innocent [43]. Compulsory boiling of milk reduced the incidence of the disease in the forces from 555, the annual average between 1900 and 1905, to 21 in 1906 [122]. In 1910 the disease was confined, so far as existing knowledge went, to the coastline of the Mediterranean [86], the spread is now world-wide [7, 292, 481] (Fig: 3).

Ornithosis

Ever since 1879 when Dr. Ritter in Switzerland observed an outbreak of sickness resembling both pneumonia and typhus in the home of a relative who collected exotic birds, house epidemics [22] and the association with parrots [176] had been recognised as the criteria of a strange disease. Vast areas of the upper Amazon have never been explored and it is possible that psittacosis, as the disease was first called, is an epizootic among tropical birds in the wild state, to which they readily succumb when herded in captivity.

Dead Brazilian parrots and their droppings [201], love birds [145], and African parrots [208] can all be infective, while

fulmar petrels in the Faroe Islands may carry infection from the fledgling stage, so that "ornithosis" is a preferable term for a whole group of epidemic viral diseases. But whereas in over 30 species of birds gastro-enteritis results, in man the disease is essentially a viral pneumonia, and in 1917 a consignment of birds confined in the basement of an American Departmental store caused an epidemic among staff and customers clearly due to inhalation [418]. Similarly ladies carrying parrots on their shoulders as part of a more than usually eccentric Parisian fashion in 1930, may have provided uniquely close contact between the human nose and the birds' execreta. But in 1897 a music professor in Florence bought a sick parrot, recently imported from the Amazon, and he and two of his family died while a child and the servant recovered. The following year a green parrot infected nine persons in one family at Cologne, only the youngest child escaping. In fact children, even though they fondle birds, seem to be much less susceptible [429], and in the English 1929 epidemic two-thirds of the cases were over middle-age, and the case mortality was noticeably higher in the older age groups. In this epidemic twelve birds caused sixty human cases, of which ten were traced to one parrot given by a sailor to the proprietor of a public house. Similar outbreaks were almost world-wide during 1929 and 1930 from Egypt to Honolulu, in every case cage-birds being implicated, often solitary ones.

Thus in Buenos Aires a dozen actors performing with a parrot all fell ill with two deaths. One might speculate that the epizootic virus underwent a highly virulent but transient mutation capable of producing severe pneumonia in those few humans, mostly adults, who contracted the disease.

CHANGES IN THE TROPISM OF PATHOGENIC MICRO-ORGANISMS

Herpes Zoster and Varicella

The possible relationship between shingles and chicken-pox has excited discussion for thirty-five years and is still undecided. Parallel epidemics occurred in Budapest in 1912 and epidemics of chicken-pox succeeding herpes zoster have been noticed in several instances [223]. Many adults with herpes give a definite history of chicken-pox in childhood, second attacks of which are excessively rare.

If one accepts the current view that shingles is in fact chicken-pox in a person sufficiently immune to localise the spread of the virus, there still remains the problem of explaining the singular spread along a sensory nerve. If a dermatropic mutant is involved the change is short-lived, for children infected by Herpes Zoster get chicken-pox not singles, and cases claimed to show both infections concurrently [236] might be chicken-pox starting with a fortuitous zonal distribution.

Mumps

When Hippocrates [2] described mumps among the boys "engaged in the exercises of the palestra and gymnasium" on Thasos, orchitis commonly ushered in the disease. While this may still be the only symptom [223] or even occur primarily in an epidemic [236], mumps is now almost always an infection of the parotids and only shows secondary spread elsewhere. Still showing a singular partiality for glands as histologically distinct as the salivaries, sex glands, breasts, pancreas and lacrimals, the virus during twenty-two centuries has developed an overwhelming specificity for the parotid gland of man.

Poliomyelitis and Polioencephalitis

In the 18 th. Century sporadic cases of poliomyelitis occurred among children, and Sir. Walter Scott, who was himself crippled at eighteen months old, described it as "the fever which often accompanies the cutting of large teeth"[270]. Underwood in 1784 ascribed the disease to teething or "foul bowels" in children aged 1 - 4, and usually among those debilitated by another fever. Shaw in 1822 saw the disease in the same age group and considered it was related to weaning. Four children were attacked simultaneously in Worksop in 1835 and ten infants all under two in Iowa in the autumn of 1841. The disease was now realised to be an affection of the spinal cord and quite unconnected with

Fig: 4. *Paralytic poliomyelitis of left leg of Ruma, a priest of the Temple of Astarte in Memphis, from a stele of the 18th. Dynasty (1580 - 1300 B.C.).*

From the Carlsberg Glypotothek, Copenhagen.

teething or weaning, but neither the infectivity, nor, far less, the epidemicity appear to have been observed [289].

Medin, at the International Congress in Berlin in 1890, first drew attention to the epidemic character of this disease, but it was still "infantile paralysis", an illness which crippled but rarely killed [55]. Further epidemics in 1911 and 1912 caused considerable alarm and the compulsory notification of the disease. Bramwell [38] pointed to its growing incidence, especially in adults, and that it had been a sporadic disease until the epidemics in Scandinavia in the 1880's.

Poliomyelitis increased prior to the "flu epidemic in 1918 and was much cited by the "epidemic constitution" theorists [183, 184] but a detailed investigation by McNalty [286] in Epsom revealed that an epidemic of 20 cases in and around a boys' school was connected through a day-boy. A rational explanation for both the epidemicity and higher age-group of the modern disease has been given by Bodian [151] and others. The polio virus is spread in faeces and most primitive people possess antibodies [48], presumably the result of subclinical attacks when young. Lack of previous exposure through hygiene can explain the present outbreaks among adults; but a further disquieting feature is the growing tendency for the virus to attack the brain giving polioencephalitis with

a consequent increase in mortality. In the Broadstairs epidemic of 1926 out of 55 cases, 32 were polioencephalitis, 17 poliomyelitis and six mild or abortive [289]. Mid-brain involvement continues to feature present-day outbreaks, thus in the outbreak among troops in the Poona area in the autumn of 1945, a marked incidence and a mortality from polioencephalitis occurred among senior officers over 35 years of age, a far cry from the day when poliomyelitis was described as a children's disease. It is difficult to see how without postulating a mutant virus one can explain the changing site for the disease.

Encephalitis lethargica

This disease is unique in having arisen, at any rate as a major epidemic within our generation, and its evolution and apparent disappearance have taken place in full view of modern epidemilogical science.

Although Sleepy Sickness had been described at Tubingen in 1872, in Italy in 1890, and in the French Army in the winter of 1915 - 16, the first authentic epidemic of fifty cases occurred at Vienna in the winter of 1916 - 17, when von Economo [113] gave the classical description of the disease as a tendency to protracted daylight sleep and marked squint lasting for weeks or months with 30% recovery, 30% invalidism, and 30% mortality. He also noted the very low infectivity which he put at 4%, and he suggested droplet infection.

Next year encephalitis lethargica appeared in France, and in March-April 1818 in England, where it was at first mistaken for botulism. Reports of 10 cases in Sheffield by Hall [181], and seven in London by Harris [186], were followed by diagnoses all over the country of cases presenting increasing stupor ending fatally [272], for instance Kennington [99], New Cross [56], six cases with five deaths at Lambeth Infirmary [276], and even a 3½ months' old baby who presented typical mask-like facies, divergent squint, stupor and spasticity. This baby being breast-fed struck the death-knell to the botulism theory, while repeated records of clear spinal fluid and the novel manifestations ruled out any infectious disease such as cerebro-spinal meningitis. Finally Buzzard [56] suggested that a new virus was abroad with a predilection for the mid-brain and brain-stem and two post-mortem examinations a few weeks later helped to confirm this view [57], which was finally proved by McIntosh transmitting the disease to monkeys in 1924 [280].

Throughout the winter of 1917-'18, the disease occurred throughout Western Europe [183], and by 1924 it had reached Japan with a mortality rise to 60%. Starting apparently on the battlefields of Flanders, within a brief seven years encephalitis lethargica had encircled the globe.

The increasing incidence and still more marked increase in mortality and mental sequelae [37,337,403,464],

caused wide concern in Britain and the appointment of a government committee to investigate the outbreaks. The investigations, involving the Ministry of Agriculture and Fisheries, and the Ministry of Health besides numerous unofficial articles by doctors and veterinary surgeons in medical journals, became a trifle ludicrous when cases were cited of two cats and two kittens suffering from lethargy and squint in a Devon village, and of three horses with catarrh at a farm at Abinger, Surrey, where a child lay ill with encephalitis [337].

McNalty [287] in 1919 and Netter in 1920 suggested that encephalitis lethargica was an epidemic and infectious disease. Preveiously the apparent sporadicity and scanty evidence of case-to-case infection had occluded its infectious character. For instance during 1919-'20 there was only one contact-case among 206 in England and Wales, and none among 414 cases from January to May 1920 in France, nor among 181 during the same period in the U.S.A. [289]. However in the celebrated Derby Girls' Home epidemic in August 1919 [281, 287] twelve children out of twenty-two were attacked with five deaths in fourteen days, and in the Mulheim epidemic in the summer of 1922 there were 28 cases in an asylum in three weeks including six nurses and three doctors. In the Sheffield outbreak of 1924 Professor Wynne[182] found contact in six instances involving thirteen cases - 40%

of the total. In this epidemic, as at Liverpool in the preceding year [402], mild and abortive cases were seen suggesting the existence of carriers [57, 193, 288] and in 1925 Da Fano [126] demonstrated the virus nature of encephalitis lethargica, and suggested its possible relation to herpes febrilis. Whether or not encephalitis lethargica was caused by a mutant of some such commonplace virus, the disease in a recognisable epidemic form has now disappeared unless we identify it with the much more benign outbreaks of myalgic encephalitis whose assaults on the Nurses' homes of several London hospitals were a clinical curiosity of the 1950's [345, 377, 400].

Smallpox

That smallpox was formerly endemic in Europe is shown by the fact that it is by far the most frequently cited disease in Saint-Simon's "Memoires", and city children grew up in Europe almost inevitably pock-marked, so that the patches and powder used by the women in the 18th. Century are said to have been introduced to disguise the scars. Hakluyt [179] tells how smallpox aided the Spanish conquest of Peru, and this extermination of the coloured races culminated in the death of 5000 natives on Guam in 1856 [396], and the complete disappearance of the Easter Island civilisation.

Following the introduction of vaccination a steady decline of the disease set in throughout Europe so that smallpox is now more prevalent in the tropics than in temperate zones, and experience shows that India is the common source of such major infection as is conveyed to this country by travel, and Massey [295] points out that the journey by air may now be accomplished well within the incubation period and constitutes a new hazard especially in a largely unvaccinated population. Widespread epidemics occurred in the Sudan in 1939 due to infection from French Equatorial Africa [350], and in Korea in 1940[396]. That there is still a risk in Britain was shown by the epidemics in 1901, 1931, 1942 [95], and 1958 [476]. The 1931 epidemic was of limited spread and low mortality and was due to an aberrant form of smallpox, Variola minor, which bred true and was identified with African Alastrian or Kaffir milk-pox, introduced into England for the first time [223].

That there was a protective effect from previous cowpox seems to have been widely suspected in country districts before the traditional Gloucestershire dairymaid enlightened Jenner. There is the unequivocal nursery rhyme: "Where are you going to my pretty maid? I'm going a-milking, Sir," she said, and Jesty, a Dorset farmer used cow's material on his family in 1774, and Dr. Bragge of Axminster possibly seven years before that. Modern vaccine lymph is the serum exudate from vaccinia vesicles propagated in calves and periodically exalted in virulence by passage through a rabbit.

Vaccinia, variola major and variola minor viruses are all morphologically and serologically identical, although the diseases are so distinct. Whether modern vaccine lymph originated in natural cowpox or artificially modified smallpox is not really known as so many passages have now occurred, including human, that the source is now forgotten. The generalised pox of vaccinia virus in experimental animals, and its necrotic lesions in chick embryos, contrast sharply with the much milder effects in both of variola major and still more variola minor. But after several transfers in both animals and eggs variola major and minor assume the pathogenic characters of vaccinia and it seems clear that all three forms are mutants of the same virus with varying virulence and animal susceptibility.

Syphilis

Unlike gonorrhoea, which has been described in Europe as a prevalent venereal disease from time immemorial, syphilis seems to have been unknown before the middle ages and most people blame Columbus for its introduction. According to this tradition, which appeals to the romantics and moralists alike, the lonely Spanish sailors found dusky companions beneath the palm trees of Hispaniola, and certainly Vincenti Pinzon, the pilot of the flagship, was ailing all the way home. Then, too, Columbus, as proof of his conquests carried women aborigines as steerage passengers, and presented

them at the court of Ferdinand and Isabella. Spanish mercenaries were fighting in the French army in Italy, and to them is ascribed the first great epidemic. This was appallingly disfiguring and reached its height in 1546 when Fracostero with amazing foresight suggested that there was an unseen "living contagion", and coined the name "Syphilis" for the new scourge.

Milder non-venereal skin diseases such as yaws and pinta, both of which are due to spirochaetes indistinguishable morphologically from T. pallidum, have their home in Central America; but bejel, another contagious spirochactal disease even more like syphilis, is common among children in Arabis, and similar milder "Syphiloids" occurred in Europe up to the 17th and 18th centuries, for instance the buccal ulcers which assailed Cromwell's army in Scotland and were described with coarse humour by the Scots as "sibbens" - wild raspberries.

It seems clear that around 1500 A.D., whether or not the New World gave it to the old, a new mutant Treponema arose which brought a devastating epidemic of syphilis to Europe, and which persists as a world-wide venereal disease to this day.

EPIDEMICS ATTRIBUTED TO VARIATIONS IN VIRULENCE

Although the more ephemeral fevers just described may afford striking evidence of mutations, for an indication

of the true power of such phenomena we must look at the rise and fall of the more prevalent infectious diseases especially among children.

Meningococcal Meningitis

Although some [396] have identified this disease with the "phrenitis" of Hippocrates and Galen [196], and the "petechial fevers" of Sydenham [260] and Huxham, the first recorded outbreak of cerebro-spinal fever was in Geneva in 1805. The characteristic winter to spring incidence was seen, namely February to April, and the typically high case mortality, unchanged until the sulphonamide era.

Following the Swiss 1805 outbreak there is an unbroken history of epidemics of the disease generally in its favourite role as a camp-follower of Wars [289, 331], although its conveyance presumed to be by "healthy" carriers was also well exemplified in Sweden in 1854. In that epidemic the infection advanced steadily across Scandinavia for seven years from the south to the north-west, beginning each winter from the point at which it had been arrested the previous summer. By 1861 4,158 deaths had occurred.

Meanwhile the American Civil War had broken out and cerebro-spinal fever was present with the army of the Potomac during the first winter, and then spread to the

confederate troops and civil population. Outbreaks continued up to 1911, when 2,800 cases occurred in Texas among crowds listening to a popular gypsy preacher in defiance of official warnings.

In 1914 Canadian troops brought a virulent strain from the New World to camps on Salisbury Plain, and cases traced to men on leave occurred all over south-east England for the remainder of the war. Thus in the 1917 epidemic three-quarters of the civilian cases were south-east of a line from the Wash to the Bristol Channel and two-thirds of Welsh cases were in Glamorgan. 6,450 cases occurred in England and Wales throughout the war, 4,238 being military [289].

Glover [162] considered that the 1917 epidemic was due to the severe winter and antecedent influenza lowering the resistance of troops, with, in addition, gross overcrowding and a high carrier rate of epidemic strains of meningococci in the population at risk. By increasing the space between beds from $1\frac{1}{4}$ to $2\frac{1}{2}$' he purged a depot of cases, but a return of overcrowding raised the carrier rate to 30% in a fortnight and brought an out-of-season epidemic in train three weeks later. He considered that a carrier rate of 2 to 5% was normal in a community and that a rise above 20% presaged an epidemic. Another potential danger noted at the time [370] was the presence of ambulant abortive cases who

had recovered in some cases within 48 hours. Between the two wars epidemics occurred on a major scale in Korea in 1934-1935, in the Japanese Mandate Islands in 1926 with 389 cases in a population of 50,000 [396] and in the Sudan in 1939 - 2,714 cases [350].

In 1940 and again in 1941 war brought major epidemics to Britain, and they showed the traditional seasonal incidence in the late winter and spring months, the slight preponderance of males, and the chief prevalence among those under five. Agglutination reactions showed the homeogeneity of the epidemic, for example in Edinburgh 116 cases in a series were Scott's group I and only one group II [222]. In the year following the British epidemic New Zealand in turn had the worst outbreak in its history with an incidence rate of 56.8 per 100,000 whites and even higher among the Maoris [396], suggesting the arrival of carriers with a strain of exalted virulence from the Home Country.

The existence of carriers and abortive cases also explains a striking feature of cerebro-spinal fever epidemics, namely the way in which even in cities most cases appear quite unconnected both in time and space, so that areas of simultaneous prevalence are separated by clear districts, and an epidemic frequently seems to spread from many small centres

rather than a single large one. Thus in the Texas epidemic of 1912, out of 2,800 cases in only 5% were two of a family attacked, and in only one instance three in a family. On the other hand the obvious infectivity of the disease was shown by the deaths of a doctor and four nurses in 1917-19 [269].

Cerebro-spinal fever has a very low incidence in inter-epidemic periods, rarely exceeding 1-2 per 1000 even in large cities, a figure much lower than the rate for the exanethemata; but given suitable conditions such as a severe winter and over-crowding of susceptibles with carriers, a correspondingly steep rise occurs, and starting in the New Year an epidemic of a new virulent mutant will reach its acme in the spring and be burnt out by midsummer having exhausted all likely contacts.

Diphtheria

Diphtheria was much more prevalent in the nineteenth century, and as the "malignant angina or cynanche" was often confused with anginous scarlet fever with which, as we now know, it was often combined [299]. The fall in death-rate began in 1880 and was attributable to a decreasing prevalence of the disease, and especially of the more severe laryngeal type, rather than to antitoxin which was only introduced some fifteen years later, or to the immunisation campaign of 1941-'45 which merely accentuated a fall already far advanced.

In Britain on the outbreak of war in September 1939 nearly one-third of the children under the age of fifteen were evacuated from the cities into the country, and this, coupled with the closure of the urban schools, was followed by a fall of over 40% in the diphtheria rates that winter among the children who stayed in town. The influx of three quarters of a million children however caused an immediate rise of 60-70% of diphtheria among the native children in the reception areas, which fell back to the normal level in six months. The epidemic prevalences occurred most irregularly, and bore no relation to the number of immigres. Stocks [413] suggested that carriers had different strains of the organism to which country children might or might not have immunity, Greenwood [172] likened the consequences to bad shuffling of a pack of cards, where one village might get more than its fair share of carriers and convalescents, while Glover [163] thought that thinning out of the children at risk by billeting with adults, and double-shift classes in small school-rooms, might explain the discrepant figures.

That the diphtheria bacillus was still ubiquitous was shown by the immediate rise in faucial diphtheria in the occupied countries of Europe [308], so that the Pasteur Institute in Brussels recorded 25,000 positive throat cultures each year, while the Institute in Paris increased antitoxin output six-fold [77]. In Lisbon laryngeal diphtheria was

still quite common, representing one quarter of cases [129], and cutaneous diphtheria appeared on wounds in the Middle and Far East [431]. It seems clear, therefore, that forecasts of big epidemics by dissemination during the war were belied only by the low infectivity of diphtheria at its present stage in the trough of its cycle.

Scarlet Fever

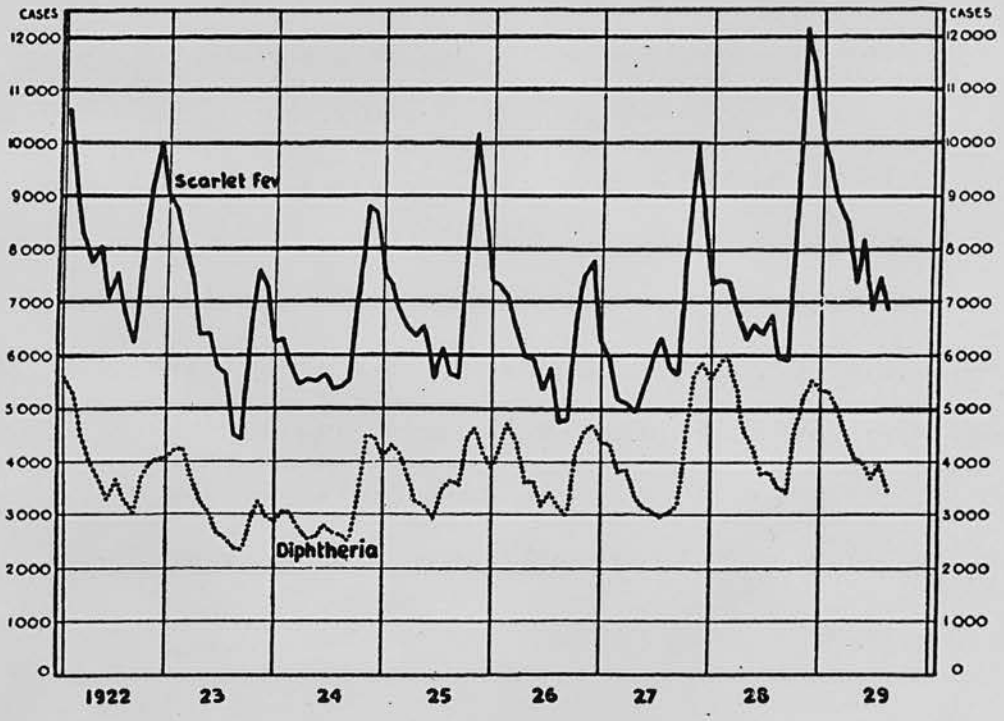
Following the demonstration by Lancefield and Griffiths of the identical organism in other diseases, scarlet fever must be regarded as but one manifestation of epidemic streptococcal infection [223]. Nevertheless it is a clinical entity, and as such has been recorded distinct from measles for four centuries, ever since Ingressio of Palermo described his "Rossania" in 1550 as "totum corpus ignitum appareat" - the whole body appears on fire. In 1574 Ballonius contrasted the disease with measles, and in 1627 Doring of Breslau described the desquamation, sore throat and dropsy ("abdomen intumescit") of a fatal case. Sydenham [260] named the disease Scarlatina in 1676, and his immense prestige doubtless led to the adoption of the name Scarlatine in France and Scarlach in Germany.

Throughout the eighteenth century scarlatinal angina was widely confused with malignant angina or diphtheria, just as the simple rash had been confused with measles in

preceding centuries. This severe anginous type of disease reached its height in the first years of the nineteenth century, waned until 1830, only to rise again the second half of the century, as "the leading cause of death among the infectious maladies of childhood" [80]. Marlborough College founded in 1843 had endemic scarlet fever almost from its commencement [263], and in Liverpool at this time scarlet fever and other infections killed nearly half of the children before the age of five, (460,370 per million). By the time a natural decline set in, in 1876, many personal tragedies had occurred, such as that of Dr. Tait, Archbishop of Canterbury, who had lost five of his six children in a single month.

There was nothing to show at the time that the decline was anything more than the usual periodic descent which would rise again in five to seven years' time. But the peak in 1878, when it came, was insignificant, and apart from some excitement over the outbreak in Hendon in 1879 which revealed a new hazard in milk-borne infection, and perturbation over hospital 'return cases' in the '90's [19, 24, 64, 98, 165, 313, 329], there has been widespread gratification at the steady drop in infectiveness and lethality of the disease (Fig: 1).

The lower mortality, which, even in the relatively severe epidemic of 1921, only reached 1.04%, no longer

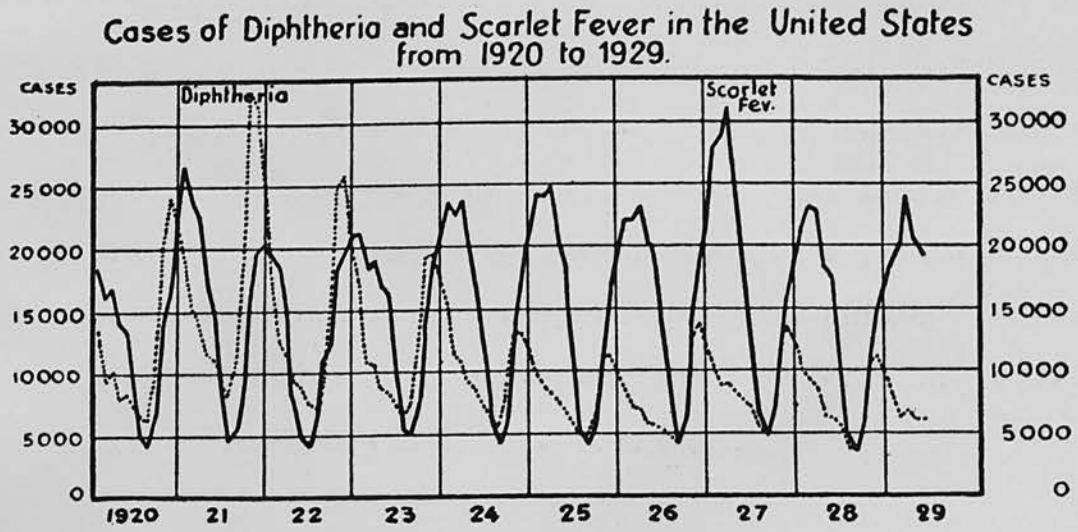


SEASONAL INCIDENCE OF SCARLET FEVER AND DIPHTHERIA IN ENGLAND AND WALES FROM 1922–1929

Fig: 5. *(After Madsen [291].)*

justified hospitalisation, and attempts were made to lower the cost of big fever hospitals with large isolation wards [39], by gargling [316], barrier nursing [378, 324], and cubicles [59], especially when it was realised that there was no danger from vectors such as fleas [183], nor, as was long thought, from desquamating cases. The main risk is, in fact, the healthy carrier [248], with a haemolytic streptococcus which can readily exalt its virulence. This is exemplified in school outbreaks which usually do not begin until the second or third week of term, suggesting a carrier in the school rather than a child returning with the infection [299], while, according to Kerr [236], out-of-season outbreaks are often due to milk-borne infection from carriers.

Among additional reasons for supposing that we are encountering a biological phenomenon such as a mutation are the following - in cities in the temperate zones, where scarlet fever is endemic, a marked seasonal wave is seen each year with its zenith in October and nadir in the spring, when the epidemic suddenly abates often without apparent relation to the weather. Moreover there is an epidemic prevalence at intervals of about five years, and it is said to present an even better marked epidemic wave every thirty years [236], especially as regards severity [223]. Although this country has been favoured by a decline in severity, Eastern Europe and the Far East still show fatality rates



SEASONAL INCIDENCE OF SCARLET FEVER AND DIPHTHERIA IN THE UNITED STATES FROM 1920–1929

Fig: 6. *(After Madsen* [291]*).*

exceeding 10%, and so appear to experience a quite asynchronous cycle. Among other curious features of scarlet fever may be mentioned the almost complete immunity of natives in the tropics, the preponderance of infection among males which may be as high as 3:1 [299], and the frequent association with anterior nasal diphtheria, chickenpox, erysipelas and erythema nodosum.

Measles

Ever since Whitelegge [463] remarked on the periodicity of measles in 1892 men have been tantalised by the inexplicable cyclic phenomena of this disease. That there are singular peculiarities in the behaviour of measles, recurring after both long and short periods, becomes obvious on the most superficial scrutiny of the statistics, but the explanation, and still less the correlation, of these changes at once leads into realms of pure speculation. Brownlee's conception of two waves, one seasonal, and the other inherent in the virus, and both nearly two years long, mutually interfering in the manner of beats, was but one attempt to fit into a single theory seasonal incidence, biennial waves, long-term cycles and the skewness of the individual wave [343]. And if for the wave inherent in the virus be substituted a mutation the theory might well fit the facts.

Hamer, facetiously suggesting that Brownlee's two different waves coexisting in London implied different sorts

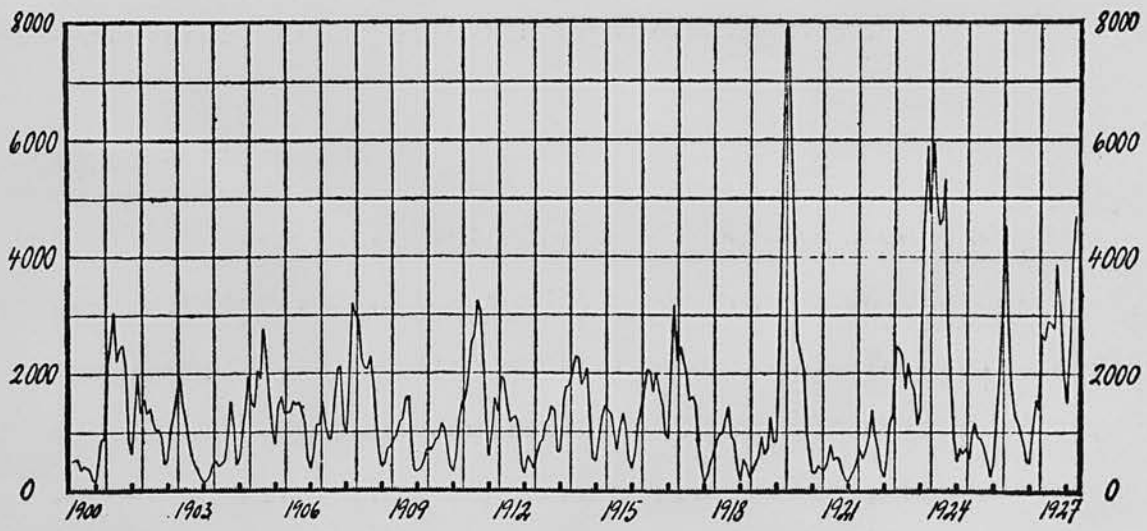


Fig: 7. The biennial wave of measles shown by Madsen's Graph for Denmark from 1900 to 1927 [291].

of measles at either end of Waterloo Bridge, may have spoken more truly than he knew, for he interpreted the biennial cycle as a periodic change in the constitution of the population at risk, which leads after each epidemic wave, to a gradual re-accumulation of susceptibles [173], and while Topley and Greenwood [174] showed that, in fact, widely spaced waves could be reproduced by slow immigration of fresh susceptible mice into a herd surviving from an epidemic, this still does not explain why although the great infectiveness has remained constant to this day, and defies all progress in preventive medicine, fatalities have markedly diminished even from the eighteenth century when the severity of measles led to confusion with smallpox. The death rate is now one quarter of thirty years ago, and the improvement cannot be entirely explained by the rise to a safer age-incidence [53].

Moreover the seasonal incidence of measles epidemics was formerly summer and autumn; but a change to late winter occurred quite abruptly in this country, in the case of Glasgow, for instance, in 1904 [54]. In hot countries such as Spain, Portugal and Egypt [223], and also in India, Burma and French Indo-China [396] where the biennial cycle has set in, the main incidence is still in the summer especially in dusty weather, for instance just before the monsoon.

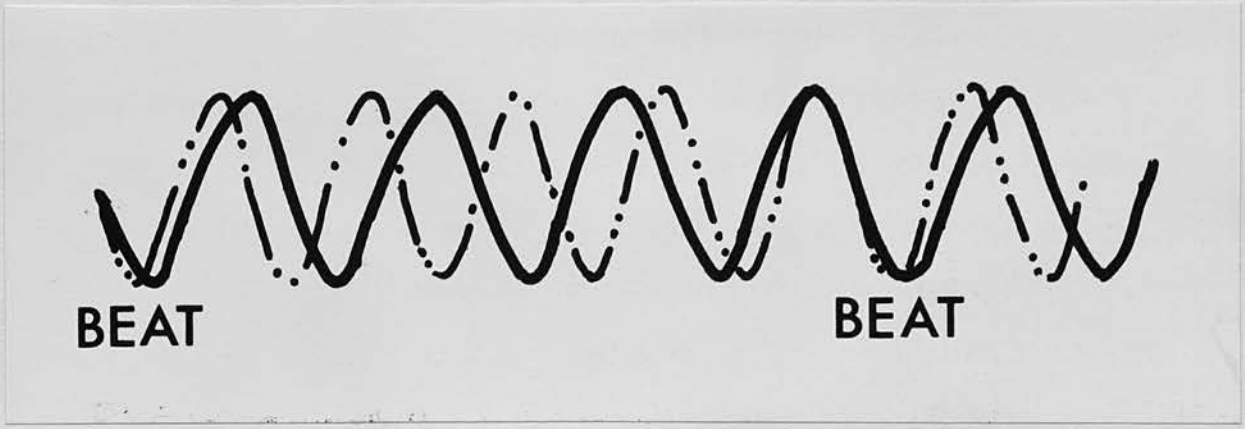


Fig: 8. *Beats are heard in music, and particularly in bell-ringing, when two notes almost, but not quite, in tune sound together. Brownlee had suggested that a similar "beat-wave" compounded of two lesser waves just out of phase could occur in epidemics as they periodically augmented each other* [343].

However both the regular seasonal incidence and the biennial wave are liable to be disturbed when measles occasionally changes its stride. Thus no epidemic appeared in 1861 or 1862 in Glasgow, in 1918 or '19 in Manchester, '21 or '22 in Leeds, nor in 1936, '37 or '38 in large areas of Australia [343] while the widespread disturbance in the cycle in 1940, while doubtless due partly to evacuation of children in Britain [218], had no such explanation in New York [414]. In the early '90's Glasgow and Renfrew had epidemics in the odd years, but in 1898 the even years received the waves which concentrated on a midwinter biennial cycle in 1904, while London changed step from odd years to even in 1918, and Manchester followed in 1920. The long-term cycle, which really amounts to a progressive flattening or increasing swing in the amplitude of the biennial waves, was elegantly demonstrated in the Renfrew statistics, the incidence waves rising uniformly between 1904 and 1908, and falling as regularly between 1908 and 1912 [343].

A curious phase of the measles cycle is the autumnal trough occurring in August and September between the consecutive minor and major measles epidemics, and alternating with the long five months sub-epidemic level occurring in other years. This is well seen in Butler's super-imposed graphs of wartime epidemics [54] (Fig: 9),

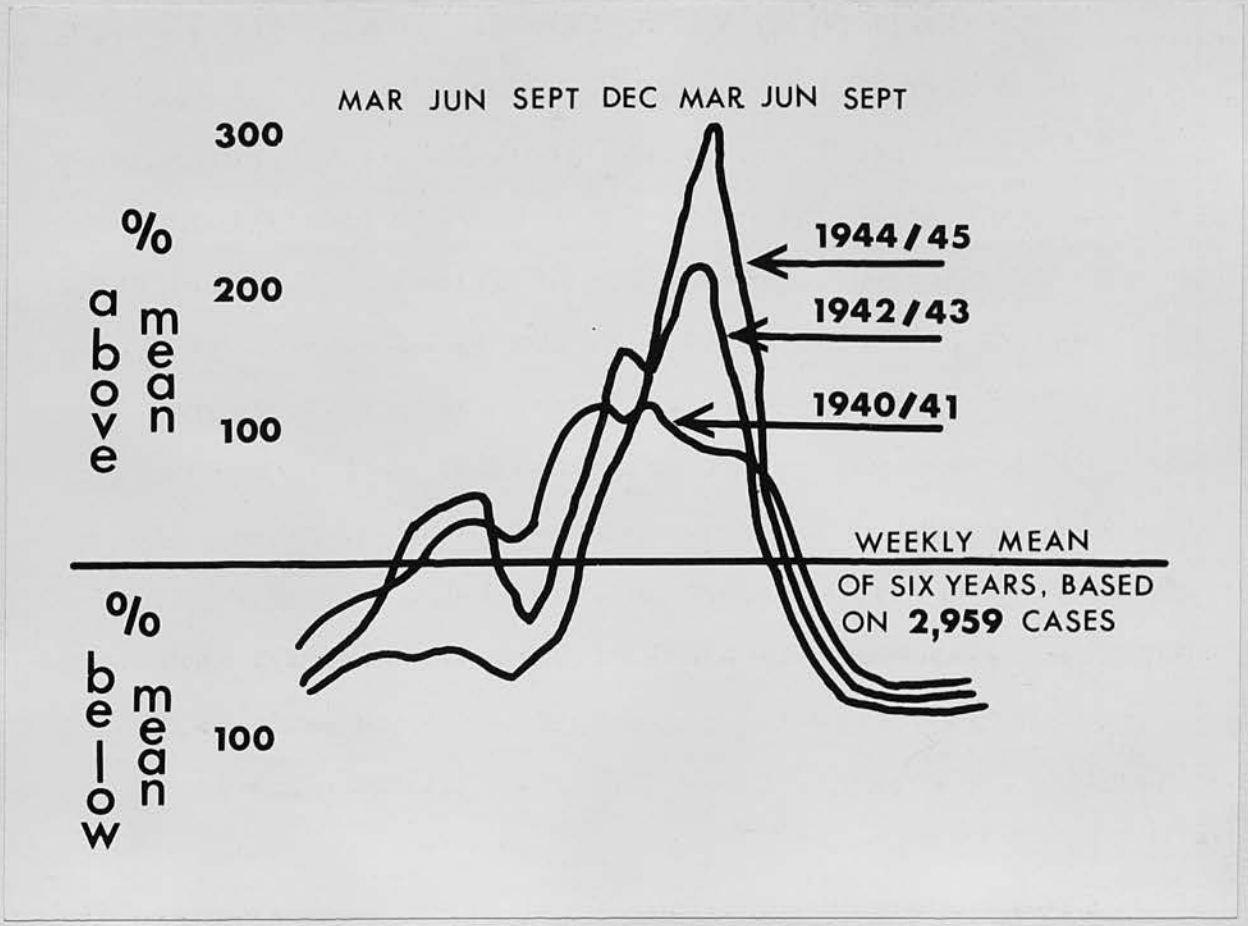


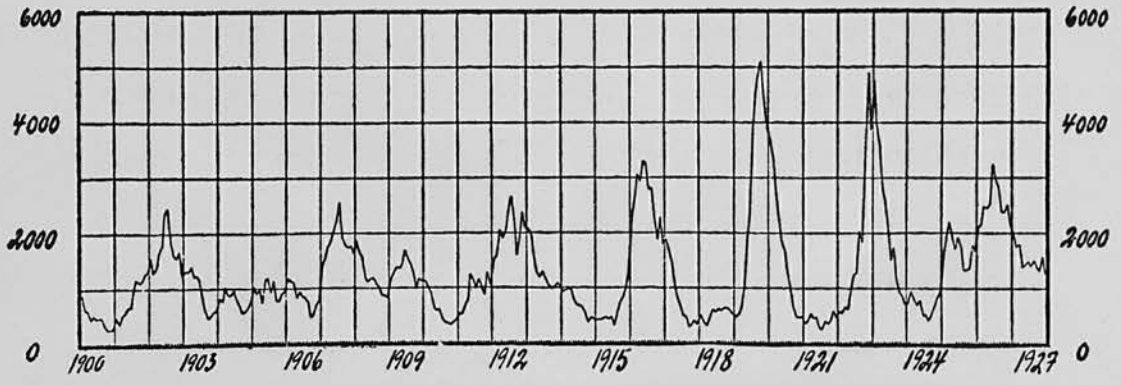
Fig: 9. *Butler's superimposed graphs [53, 54], of three epidemics of measles shows the remarkable way in which the successive biennial waves follow a common pattern.*

and Stallybrass [401] has remarked that this deterrent effect on the infectivity is also seen in influenza and may occur in September when the dispersability of scarlet fever and diphtheria are reaching their height.

Whooping Cough

In the winter of 1845 Farr recorded a big epidemic of whooping cough occurring in London in conjunction with measles, influenza and typhus. This close association between whooping cough and measles has been noticed on many occasions since, especially in the large cities of Europe where the two diseases are epidemic every three years during the winter and spring [236] (Fig: 10). Remarkable changes have occurred in the rise and fall of the epidemic waves, the troughs sinking so low by 1931-'40 that a large part of the inter-epidemic periods were free from deaths, and the overall death-rate between 1921-'30, while still exceeding that of both scarlet fever and diphtheria combined, was but a third of that half a century before [223].

Whooping cough has a predilection for girls and twice as many epidemics occur in their schools as in boys' [299]. Moreover 99.3% of cases notified in four London boroughs were children under the age of fifteen [412], and as, even on previously unexposed islands such as Samoa, Guam and the Pelaus [396], it is the native children who were mainly



SEASONAL INCIDENCE OF WHOOPING COUGH IN DENMARK

Fig: 10. The triennial wave of Whooping Cough shown by Madsen's Graph for Denmark from 1900 to 1927 [291].

affected, one must conclude that adult resistance to the H. pertussis is a change in the parasite-host relationship and does not merely depend on previous subclinical attacks as might be supposed.

SETTLING IN EPIDEMIC CYCLES

Madsen [291] working in Copenhagen in 1937 used the homogeneous and highly organised population of Denmark to study cyclical changes in the common infections there. His graphs cover a decade and show most elegantly how remarkably uniform the recurring waves are both in wave-length, that is the duration of the epidemic, and in amplitude representing its incidence. But they also show a much more significant feature, that there are distinct groups of epidemic diseases.

Group 1.

First there are, for example, scarlet fever and diphtheria which have settled down over the centuries to a strictly seasonal incidence with maxima in November and January respectively, and minima in July. Year after year they follow this seasonal channel unchangeably, so that they are in a sense more endemic than epidemic. Madsen's graphs for these are shown (Figs: 5 & 6).

Group 2.

By contrast there are those diseases which also recur rhythmically but whose waves of infectiousness quite overcome the seasonal variations. Measles comes round every 2-3 years and whooping cough every 3-5 irrespective of the season, so that while the maximum mortality for whooping cough may be, as if often stated, in the spring, the maximum incidence may occur at any time of the year (Figs: 7 & 10).

Group 3.

Finally there is the peculiar example of influenza, whose fluctuations appear so asynchronous on a graph that they can only be due to several superimposed lesser waves alternately potentiating or opposing each other (Fig: 11).

Madsen attributed his waves to alternating variations in human resistance, citing Simpson and Clayson's [397] observation that pus in tuberculous glands is greatest in March, and his own discovery [9] that the response of animals to diphtheria toxin is greatest in winter. But a much more likely explanation is a variation in the parasite, and, if we accept this, it is hard to resist the conclusion that recurring mutations are at work, and are more drastic in some species than in others. It is the hypothesis of this study that this is precisely what is happening. It is suggested that there is a gradation in the epidemicity of

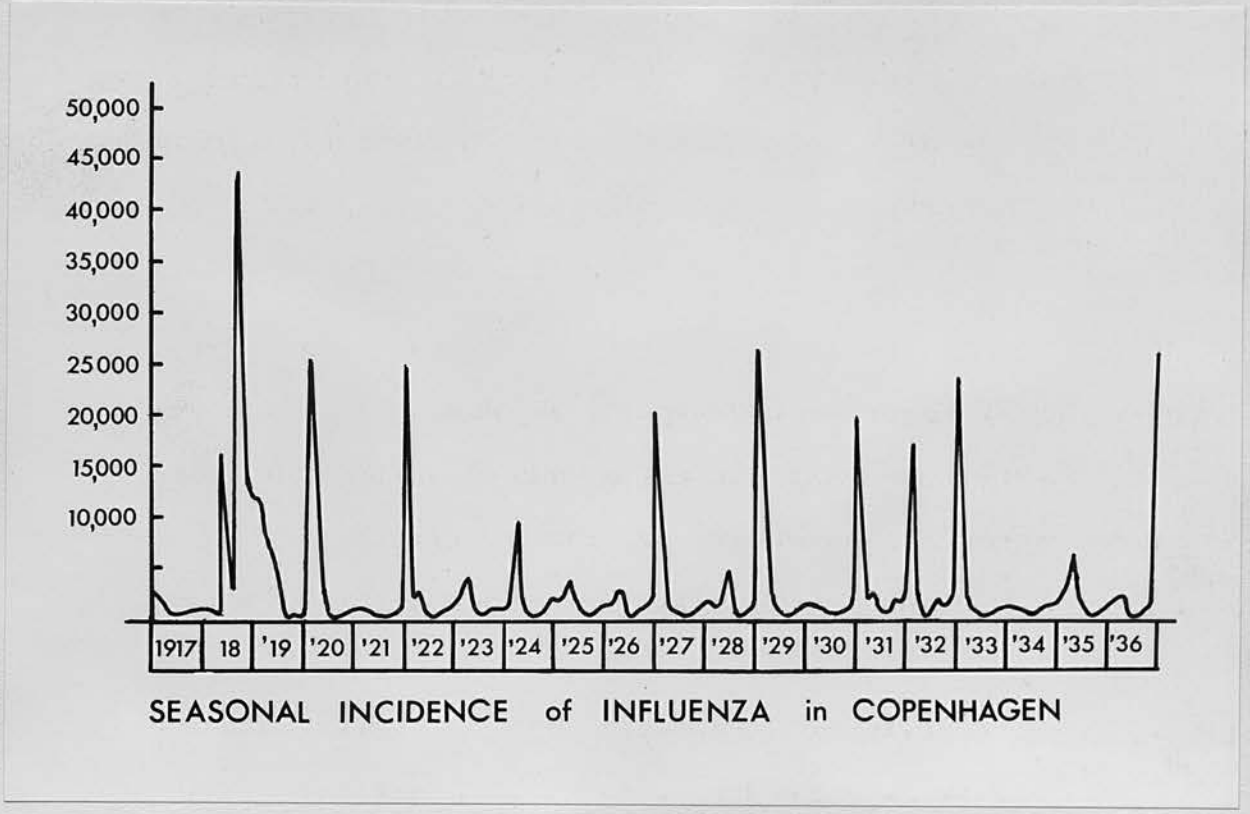


Fig: 11. Incidence of Influenza in Copenhagen, 1917 - 1936 (after Madsen [291]).

infectious diseases which depends on their mutability. Measles and whooping cough, and still more influenza, are still very actively mutating, and their abrupt transitions cause a greater impact on the individual and on the community. Such diseases as diphtheria and scarlet fever long ago became more quiescent, and, while still mutating, they have reached a stage of greater stability. If successful parasitism is the objective of the species then equilibrium with the host must be the measure of its success. But perhaps the best evidence is, by contrast, the extreme mutability and therefore instability of influenza which is described in Chapter 4.

CHAPTER 4. INFLUENZA PANDEMICS AND IMMUNOLOGICAL DRIFT

HISTORICAL INCIDENCE

Although this disease was ironically referred to as the "Newe Acquayantance" at the court of Mary Queen of Scots in 1562 [146], there seems little doubt that epidemics of chills, coughs, langour and fever identifiable as influenza have recurred for over a thousand years since A.D. 877. Precise descriptions of the 1657 outbreak were left by Willis, of that of 1675 by Sydenham, 1729 by Huxham and 1767 by Heberden, so that Creighton [80] remarked that influenza almost alone among infections diseases had remained true to its type. But although this persistent success has required no clinical change, remarkable mutations of the antigen have occurred to evade the antibody defences. Thus 1847 ushered in the era of modern pandemics recurring at 30-40 year intervals, in which the virus showed that it had evolved to complete supremacy of infective power with a toxicity almost unrivalled by any other infectious disease. This pandemic lasted six weeks and at its height in the first three weeks of December 1847 the death rate for the elderly rose by 247% due to influenza and its complications of bronchitis and pneumonia.

After 1850 the disease almost disappeared until the next great pandemic of 1889-'92. Once again a high death-rate

from pneumonia was seen and for the first time the epidemic revealed the successive waves separated by short intervals characteristic of modern influenza. It was realised that miasmatic and climatic causes were no longer tenable, and that the disease had spread from person to person by direct contagion, coming to this country from China and Russia, and continuing westward to America and Japan.

In contrast to the previous inter-pandemic period the next twenty-five years were marked by repeated minor outbreaks culminating in the astonishing pandemic of 1918-'19 which exploded almost simultaneously all over the world. In a few months influenza killed 10 million, more than the greatest war in history had been able to achieve in five years, and the disease fell as heavily on Samoa and other Pacific islands as on nations exhausted by the war [396].

Almost everywhere a characteristic tripe wave was seen. Thus in Great Britain [123] the epidemic began at the end of June with an abrupt onset of 3-day fevers, continuing, mostly in the younger population, for six weeks. At this stage the fever was followed by a rapid convalescence and low mortality. But in October a second wave of much graver illness arose, and starting insidiously it lasted for twelve weeks. Three-quarters of the deaths occurred in this wave, often due to secondary broncho-pneumonia [6, 321]

heralded by heliotrope cyanosis and haemoptysis on the third or fourth day. In February 1919 the third phase was seen as a final wave of intermediate virulence lasting eight weeks [327].

After 1918 influenza resumed its former role in minor outbreaks [210] (Fig: 11) which, Brownlee [42] asserted, tended to occur in a 33-week cycle or its multiples, preceded in the previous week by pneumonia and a fortnight earlier by bronchitis, diseases which, he suggested, had become ancillary to influenza in the outbreak of 1889. The seasonal incidence in January and February was seen in the British epidemics of 1920 [327], 1936-'37, and 1940 [279], and in Australia in 1939 [48], but Burma and Siam experienced annual outbreaks in the cold season following the November north-east monsoon.

In 1931 Shope showed that Swine Influenza was due to simultaneous infection by a virus and Haemophilus influenzae suis, neither being sufficient alone. However no such symbiosis has been found necessary for the human disease, a virus, now known to be one or other of two types 'A' or 'B', sufficing alone. Whereas both types are clinically similar being primarily respiratory infections with a 2-day incubation period, and probably only a single day of infectivity, 'B' is a milder disease. A is on a 2-3 year

cycle, B on a cycle twice as long. In addition however to these regularly recurring waves there is a much bigger cycle accounting for worldwide pandemics nearly half a century apart.

Thus early in 1957 Chinese virologists in Peking reported a new strain of A. The 1782, 1830, 1889, and 1957 epidemics all originated in China and the World Health Organisation, of which China is not a member state, believes that the disease is harboured there in horses, pigs and wild animals [226, 478]. The 1957 virus was found to be infecting horses and pigs in many countries, and Swine Influenza [395] itself first reached prominence during the 1918 human epidemic even suggesting that it had arisen from the human disease.

The new 1957 virus was found on arrival in Singapore to be immunologically distinct from all previously isolated strains, and it was realised at once that the whole population of the world was potentially susceptible. As expected Strain A/Singapore/1/57 as it was called spread round the world westwards in 2 waves reaching the British Isles in Autumn '57 and January '58 where 3,000 deaths were recorded. The confident predictions concerning the 1937 epidemic were based on new knowledge of the structure of influenza virus. The inner ribonucleic acid core is fixed

and distinguishes the virus itself as 'A' or 'B'. The surface protein which is concerned with the initial infectivity is immunologically constant for that one single epidemic only; but subsequent changes are of minor character until a major variation occurs, roughly once in a decade. Thus the virus of the spring of 1957 was unmistakeably 'A' but the surface antigen marked a sharp break from those of the previous large epidemic of 1946. It was realised that the entire world might well be at risk, and that Europe and America could count on up to 6 months delay to prepare their vaccines, but that Australasia would be attacked imminently, and this in fact happened. Many millions were vaccinated, particularly in the U.S., using inactivated virus grown on the allantois of chick embryos. It is a measure of the exalted virulence that pandemic 'A' flu infects the allantois as well as the amnion; ordinary inter-epidemic 'flu needs serial passage before this is achieved.[52].

Observations in Australia [235] showed that those over 25 years old had some degree of immunity, suggesting that the 1935 epidemic, a quarter of a century previously, had marked the appearance of a new variant virus. But neither infection by this nor by the 1946 strain could ensure full protection in 1957. On the other hand Mulder and Masural [322] were able to show in Holland that survivors of the 1889-92

epidemic still living were not only spared but gave serologically evidence suggesting that they had encountered virus A/Singapore/1/57 before [52]. This affords an implication bordering on the bizarre - that Flu virus is so mutagenic that in less than a century, it has exhausted the available permutations even of major variants and inevitably repeats itself.

Hirst [197] by serological absorption tests showed in 1952 that seven distinct types of Influenza A had temporarily established predominance in the previous 20 years, and Burnet later detected almost annual changes suggesting that there was very frequent discontinuous mutation. Each mutation proceeds from the former ones so that immunity will confer protection also to the earlier strains; but not to future ones unless back mutation occurs. Mulder's immune octogenarians suggest that such a back mutation occurred in 1957 and that therefore a long-term cycle may well exist in the antigenic variation of Influenza A. Similar cyclical changes are still more likely among the minor mutations and could explain the biennial frequency of interepidemic 'A' and the 4-yrly waves of 'B', while superimposed mutations in virulence, and transmissability which are always occurring [226] must add further esoteric effects. There is some experimental evidence too that genetic interaction can occur in Virus A to increase its

mutability. Burnet Fraser and Lind [49] mixed large amounts of the Melbourne Epidemic virus with another strain in chick embryos, and claimed recombinants emerged with character derived from both. Suffice is to say that recent outbreaks continue to display the cyclical phenomena of this historic disease and that the 1959 Influenza of 'B' type showed serological resemblances to that of 1954 [214, 420].

GENETIC DRIFT

The principle of genetic drift is generally accepted, whereby successful new characters replace the outmoded old in a community by elimination and gradually become the normal features. A similar 'survival of the fittest' would appear to be spreading with the 'flu viruses throughout ages, and Burnet [52] has suggested that they show an Immunological Drift analogous to the genetic drift of higher species.

Hirst had disclosed the succession of antigenic patterns evolved by Flu virus A, each in turn sweeping the world. Jensen [221], however, showed that there were three marked discontinuities so that in the 40 years 1918 to 1957 there was a particularly abrupt change of the virus in 1929 after 11 years and again in 1946, with a final decade ending in the 1957 epidemic. He suggested that 4 distinct families of virus succeeded each other at these times, and persisted

for one or two decades. Even if we accept this view, that such major variations succeed each other, it would still appear that minor variants similarly replace their predecessors almost annually, and are no less successful in spreading throughout the world. This is borne out by the Davenport phenomenon [89], that the first 'A' flu epidemic in childhood largely determines one's immunological response throughout life. Thus at present (1962) only those over 33 yrs. old have the antibodies to the pre-1929 epidemic strains, and only these over 12 to that which followed, while present-day children should have the 1957 "Asian" strain as their dominant 'flu immunity. By the Davenport principle each new epidemic will supersede all those previous to it, conferring immunity to itself and also afresh to the strains previously encountered, and thus preventing these strains returning to the population a second time. Eventually all the permutations having been exhausted, a major mutation of a lifetime ago will be repeated so that, as Mulder found, the very elderly are confronted by a familiar strain once again.

Owing to its intense infectivity and droplet spread Influenza viruses have potentially the whole world for their arena; and certainly all civilisation will be successively assaulted by each new variant strain as it rises to replace the last. There seems no real reason to doubt that a similar continuous evolution based on successful mutations

is operating with other parasitic organisms, and there is evidence that this is so, for example with the enteroviruses particularly the ECHO infections [230] and anomalous polio strains. But whereas 'flu by its rapid spread can always find outlying human communities in which its novel forms can secure a new foothold, the enteroviruses depending on faecal transmission and at the mercy of all modern man's sanitary improvements, will only rarely mutate sufficiently to succeed in the same population as that infected already by the parent virus. Such a new variant is suggested by the worldwide spread in the 20th century of paralytic polio occurring in epidemic form. But in general the picture with the enteroviruses is not one of world pandemics as with 'flu; but of localised out-breaks in which dozens of variants. - ECHO viruses, Coxsackie and true polio can co-exist without any one assuming temporary world-dominance.

PART II.

RHYTHM IN BACTERIAL MUTATIONS

CHAPTER 5. THE NATURE OF BACTERIAL MUTATIONS

The ability of a proportion of bacteria in a strain to withstand a drug depends on an adaptation. In theory [425] there are two such methods of adjustment to the adverse environment. In "phenotypic adaptation" changes in the cytoplasm occur in at least a few cells, such as the production of the enzyme penicillinase whereby the toxic drug, in this case penicillin, is neutralised. These phenotypically adapted cells can come to outnumber their sensitive counterparts as the drug continues to act; but they do not transmit the new ability genetically, and removal of the stimulus is believed to lead to immediate loss of the characteristic.

In "natural selection" or "genetic adaptation" the chance occurrence of naturally resistant mutants enables the race to survive, and by overgrowing the sensitive population, as it is exterminated by the antibiotic, a new drug-resistant strain appears capable of taking a permanent place in evolution.

Much discussion has centred on an apparent distinction between two patterns of drug-resistant mutation. With penicillin and most other antibiotics bacteria serially subcultured with increasing amounts of drug gain resistance in a regular step-wise manner. With streptomycin the

increase in resistance can be abrupt, and, it is often said, of unpredictably large or small degree. In fact, however, as experiments in this thesis will show, there is no real distinction except in the degree of mutation rate. First-step and subsequent mutants of all degrees can be shown alike to streptomycin and other antibiotics; but with some drugs, and particularly streptomycin itself, they all occur with much greater frequency.

While mutations confirming drug-resistance to insecticides seem to be possible to all insects [25], this is not so with bacteria where certain species such as the Haemolytic Streptococcus have failed through many thousands of generations to produce penicillin-resistant mutants [101].

Natural Selection is a preferable term to genetic adaptation, which can have the unfortunate connotation that creatures are deliberately adjusting not only their own destiny, but that of their progeny. This of course never happens. Either a species fits its surroundings or it does not. If it does not it will fail to reproduce and the race will vanish [115].

This process of screening occurs throughout nature and examples of such apparently intentional sorting, which are quite as remarkable, occur among inanimate objects

subjected to physical forces regularly applied. An extreme example is Chessil Beach in Dorset where the pebbles are accurately graded for 18 miles from ¼" to 6" in diameter apparently by tidal drifting [119].

With living creatures there will be mutations and variations constantly occurring, and in a ceaselessly changing environment these new combinations arising from the astronomical numbers produced by living protoplasm will be needed merely to maintain a stable equilibrium. "Natural Selection" or "Adaptation", call it what we will, is merely a demonstration of a game of chance in which a species, by its mutants, survives the ever-changing conditions through time.

Microbes evolved early in the world's history, and where there has been no call for any change in specific characters these have been fantastically immutable [61]. Thus a dental abscess has been found in a dinosaur skeleton 100 million years old, and cocci occur in pre-cambrian limestone five times as old again [185]. Staphylococci have kept their cluster formation of some twenty organisms for at least sixty centuries as shown in serial sections through the gums of an Egyptian mummy (Fig: 12). Similarly the production of coagulase is evidently a very fixed characteristic of virulent staphylococci. Strains of Staph. albus isolated

Fig: 12. *Transverse section of alveolar margin of tooth of Egyptian mummy of approximately 4000 B.C. The cluster of Staphylococci near the centre was seen in several contiguous serial sections.*

from urinary infections may not possess this feature, presumably because their parasitism was on the surface of, for instance, the bladder mucosa. Four such strains injected intraperitoneally into mice produced a purulent ascitis still without invoking coagulase production. It seems likely that possession of the enzyme would require a mutation only likely to persist where invasiveness is demanded of the parasite.

THE MECHANISM OF SPONTANEOUS BACTERIAL MUTATIONS

Hugo von Mohl in 1846 wrote: "The remainder of the cell is more or less filled with a white fluid having granules intermingled in it, which fluid I call protoplasm". This description applies to all living cells, and bacteria are no exception; but in them the granules of protein and ribonucleic acid are so numerous that they mask the sharp distinction between nucleus and cytoplasm which we see in higher organisms. If the ribonucleic acid granules are dissolved by hydrochloric acid, nuclear bodies can always be found showing the staining characters of desoxyribonucleic acid (DNA), the characteristic component of true nuclei. Unlike nuclei, however, the nuclear bodies of bacteria divide asexually and not by mitosis.

Normally bacteria divide by simple fission of the nuclear body, each half retaining round it its half of the

cytoplasm, and the resulting progeny maintain their characters unchanged through generation after generation. In the nuclear body are genes believed to be molecules of DNA, which determine these unchaning features, and there must be fully a thousand, each reproducing itself and controlling a distinct substance or enzyme figuring among the inherited characters. Presumably at meiotic cell division the genes align themselves along the axis of a chromosome in regular order, ready to split into two parallel sets, one for each daughter bacterium.

If a gene becomes altered displaced or lost, one or both daughter bacteria will be mutants with a new characteristic which they thereafter transmit to all their offspring until some further mutation occurs, or still more rarely, until a "back-mutation" reverses the first. Any gene may suffer such a spontaneous mutation, and the rate for any one gene is probably constant, although the different genes differ widely from each other. Thus Duguid has pointed out that a large bacterial colony, resulting from say a thousand million divisions, will have thousands of mutant cells, most due to genes mutating at perhaps 1 in 10^4 per generation, but a few to those with only 1 in 10^{10} mutation rate [107].

The mutant cells on the scale seen among bacteria could be lethal to a higher animal if they flourished and colonised its tissues. Is the incompatability of homografts an example of pre-adaptation as Medawar [297] has suggested? In mammals, by making a mother shed the foetus and placenta in their entirety, it can prevent the ongrowth of foetal tissues which could become hyperplastic. But the incompatability of homografts also occurs in fish and reptiles who originated 300 million years before the first mammals.

Besides the actual phenomena of mutation the existence of an 'ecological niche' appears to be essential to evolutionary changes, because unless the new forms have the opportunity to mix and exchange genes in relative isolation, they will not dominate a species. Such a niche occurs in hospitals where nasal carriers among the staff and actual patients enable the relatively rare penicillinase-producing staphylococci to multiply in relative isolation. Gerard has called this micro-evolution [155]. Thus streptomycin-resistance is due to micro-evolution by a high mutation rate in a single host while penicillin-resistance is a slower micro-evolution due to a low mutation rate among populations of bacterial strains in the ecological niches of hospitals.

As long ago as 1928 Griffiths [441] showed that a transfer of genes occurred between two pneumococcus variants A and B. He mixed dead A and live B variants and injected them intraperitoneally into a mouse. Subsequently living A types were found in the mouse, showing that transformation has taken place by A introducing genetic material into B. This transfer of protoplasm can also occur by transduction, in which a bacteriophage virus successively invading A and B can remove protoplasm from one to the other.

Thus in addition to the classical sexual union of higher animals in which two partners contribute half-shares to the genetic constitution of their descendents [46], there are at least two other possible processes for maintaining a species in nature, transformation and transduction, and both may occur among bacteria [307].

Another relatively rare mechanism giving mutations which appear to be heritable, is lysogenic conversion. This occurs when a parasitic bacteriophage contributes genes to its host, and unlike transduction the entire infected population of bacteria will exhibit and transmit the new character; but only, of course, during the sojourn of the parasite. Lysogenic conversion is known to explain toxin production by hitherto avirulent diphtheria bacilli.

The most remarkable of all phenomena conferring new inherited characters is conjugation between bacilli. Certain E. coli when in actual aposition fuse sufficiently for genes to stream across from one cell into the other. The recipient, now at least partly a diploid cell, undergoes a reducing division to yield two haploid bacilli with characters derived from both parents. The fertility factor which predisposes to this phenomenon is itself thus passed, and also that controlling colicine production, but for other genes this must be quite exceptional, although Xalabarder by studying serial electron photomicrographs has suggested that it is the rule, rather than the exception as is generally supposed [483 - 488, 490, 491]. This will be further discussed in Chapter 12.

Not only are all these four mechanisms probably rarities, they are also necessarily haphazard in their incidence, and we are looking, of course, for a regular cyclical phenomenon capable of explaining the systematic wave-like rise and fall of epidemic diseases. This thesis is intended to show that spontaneous gene mutations by their essential constancy could offer a mechanism which would fulfil this role.

The alternating "phase variations", among, for example, Salmonellae, by which fimbriae and flagella are

lost on solid media only to be regained in liquids, have been regarded as arising from exceptionally prolific mutations; but whether they are indeed genetic seems doubtful.

ARTIFICIAL INCREASE OF MUTATION RATES

Since natural selection may first disclose a mutation, it is tempting to suppose that the environment was itself the cause of the change rather than merely selecting organisms from the results. Certainly there are mutagenic agents in the environment such as radiations and chemicals which can increase mutation rates thousands of times, and some are used, for example in the propogation of antibotic-yielding moulds. But mutagens cannot do more than increase the overall mutation rates for all the exposed genes. They cannot promote one selected character, although the same agent might presumably provide also the environmental conditions for subsequent selection.

THE CONSTANCY OF BACTERIAL MUTATIONS

It is now generally appreciated that mutants must be quite frequent among bacteria because of their enormous multiplication, and among the best known are the dwarf 'L-forms' in many species. These variants, often only 200 mμ

in size [114], are said to be stimulated by antibiotics [296] and so may be part of a cyclic change to withstand adversity[453] though Tulasne and Brison have also suggested that they persist [452].

The species Escherichia coli mutabilis is remarkable for its instability, and Zamenoff [500] has shown that the strain contains, in fact, two main mutants, one long-chained and less active than the discrete type, which alternate every 1,000 to 5,000 divisions. There have been occasional calculations of the frequency of various other mutations: thus Witkus [473] observed that white colonies appeared among the yellow of Sarcina lutea in a fixed proportion of 1 in 40,000, and Elek [118] found that streptomycin-resistant mutants in a strain of Staphylococcus aureus were constant at 1 in a 100 million before the drug itself disturbed the equilibrium. Illemann-Larsen showed more precisely that matural resistance to 4 g. of streptomycin distinguished 1 colony in 14,600 of a strain of tubercle bacilli[209]. At a medical conference in Moscow in 1962 pale colonies were described among the red in Serratia marcescens appearing as mutants with a constant rate of 1 in 10^{-4}.

When we look for reasons why such counts have not been made more often, we find that the apparent simplicity of the calculation is misleading, because, in fact, a single

isolated character which is also conspicuous like the colour of the colony is exceptional. Much more commonly the mutant is one of many, differing either in degree or controlling quite distinct characters.

THE HETEROGENEITY OF A BACTERIAL POPULATION

Thus even such an apparently uniform characteristic as the drug-resistance of Pseudomonas species is, in fact, possessed by only some of the bacilli [191]. Welsch [462] showed that Staph. aureus No. 126 had two distinct mutants both resistant to 5 μg., of streptomycin but one with full-sized colonies, the other with much smaller ones. Szybalski [423] in a classic experiment using penicillin gradient plates seeded with Staph. aureus N.R.R.L. No. 313 found among its resistant mutants 1 in 20,000 which not only survived themselves; but also allowed a halo of faint general growth around. He showed that these were, unlike all the other resistant mutants, penicillinase-producers rather than outrightly insensitive, in fact they only just withstood penicillin themselves. They differed, too, from other penicillin-resistant mutants in being as fast-growing and virulent as the parent sensitive strain, so that clinically they could eventually become dominant even if initially they were the rarest of the rare.

PROGRESSIVE MUTATIONS

Drug-resistance is not, however, a mere matter of the simple accumulation of these insensitive mutants. Thus Tompsett [439] explained relapses in his tuberculous cases by observing that bacilli resistant to 1 μg., isoniazid had become a third of their Mycobacterial population; but Wallace and his colleages in Edinburgh [456] showed in a similar series that in spite of back mutations there was a steady rise in absolute resistance reaching the maximum strength tested (50 μg.) in less than five weeks. It seems quite clear that such mutations are not only cumulative but also progressive and that, as Demerec suggested with penicillin-resistant staphylococci, higher potency genes emerge at every step. Experiments with specific strains of Escherichia coli will be described to show that drug-resistant mutations are not only regularly occurring but that these in turn are the progenitors of regularly occurring resistant mutants of higher and higher degree. We will then go on to study similar regular mutations recurring among the tubercle bacilli multiplying in an individual patient as an ecological niche over months or years, and finally we will consider the implications of such periodically emerging mutants in the cycles and evolution of infectious disease. The particular mutants chosen throughout these studies were those conferring resistance to streptomycin for the reasons given in the next chapter.

CHAPTER 6. DRUG-RESISTANCE AS A VISIBLE MUTATION

To a biologist all living things are the products of evolution, and to a bacteriologist there is the additional fascination of watching his organisms pass through a score of generations in a single day. Among the prodigious numbers of organisms involved, mutations are sufficiently numerous to be seen happening before our eyes. A drug-resistant mutation is only one of these many; but it is conspicuous, and may provide a new tool for studying the frequency of bacterial mutations in general.

Drug-resistance may appear during any chemotherapeutic regime, and is almost certainly due to the selective breeding of a few resistant bacteria present in the original stock of infecting organisms [246]. Thus in prolonged therapy as in tuberculosis the gradual onset of resistance in cultures isolated during the first few weeks, the increasingly rapid progress from the second or third month, and the final attainment of an apparently permanent level of high resistance provides in many cases strong presumptive evidence that we are dealing with the cumulative effects of multiplying variant bacteria.

The experiments which will be described in this thesis suggest that this selection of variants is indeed occurring,

though it would appear from the continuing superior count of the control plate that not all the progeny remain genetically stable. If it could be shown that the frequencies of occurrence of drug-resistant mutations are constant for any one strain of a species, both initially, and as regards the progressive mutations producing a cumulative effect in successive generations, the knowledge could be of clinical significance.

Garrod [152] has pointed out that apparent superinfection during antibiotic therapy could be due to the emergence of resistant organisms already present, to the drug actually stimulating them, to disturbance of the natural antibiotics which supposedly maintain their equilibrium, or to a new opportunist organism which chances to find the vacuum. Of these much the most likely is the first: resistance due to the multiplication of an existing mutant clone. Eagle [111] in the case of penicillin-resistance suggested that several alternative mechanisms could confer such survival. They could produce penicillinase extracellularly, or intracellularly, or avoid binding the drug altogether, in which case, as he showed, unaltered penicillin would remain present.

The team-workers of Finland [134, 136] and Barber [16] have shown that penicillin-resistant staphylococci have

multiplied from a few variant strains which originally happened to possess the neutralising enzyme penicillinase. The actual individual mutation from penicillin-sensitivity to penicillin-resistance has not yet been observed and it must be of exceedingly rare occurrence. By contrast, resistance of staphylococci to the other antibiotics appears to have come about by current mutations. Evidence of these mutations is seen clinically most often with streptomycin, and less frequently with erythromycin, novobiocin and the tetracyclines, while with chloramphenicaol they seem to be rare [460].

This apparent rarity of chloramphenicol-resistant staphylococci is not, as might be supposed, related to the amount prescribed [265, 400]. In spite of the heaviest use of the antibiotic the phenomenon still holds true [377, 476], and is partly due to a low mutation rate [166], but still more to a relatively high back-mutation rate restoring the staphylococcus to its original status of chloramphenicol-sensitivity [63, 166, 234]. This is readily seen in surgical wards, where stopping the use of chloramphenicol allows the rapid return of sensitive organisms [157]. Cessation of penicillin, by contrast, confers no such immediate benefit because the penicillin-resistant strains must be actually replaced by the slow regrowth of sensitive stocks unhelped by mutations. The other antibiotics, streptomycin and the tetracyclines, lie intermediate in this respect between these two extremes.

Since Rammelkamp and Moxon [355] first postulated the existence of these mutants there has been much speculation as to their origin, and Fairbrother [125] suggests that penicillin-resistant staphylococci represent a survival from a primitive or saprophytic type. Inherently high drug-resistance is certainly more common among some of the less parasitic organisms such as Staph. albus, and species of Proteus and Pseudomonas, and it has even been found in Staphylococci from the jungle of New Guinea [375].

The pattern of drug-resistant mutations varies with the species of organisms as well as with the antibiotics [416] and their dose [170] a great deal more than is generally supposed. There has never been an authentic report of any strain of true haemolytic streptococci gaining resistance to penicillin, chloramphenicol or erythromycin [23, 101, 135, 359, 376], though a few strains have achieved tetracycline-resistance [273]. H. influenzae has remained consistently sensitive to chloramphenicol [376, 502]; but species of the genus Proteus resistant to this antibiotic are increasingly encountered [158].

The enormous reproduction rate of bacteria would allow even the rarest variants to replace the normal population, strains of Staphylococcus aureus resistant to penicillin and other antibiotics are now notoriously prevalent [17, 18, 124, 131, 137, 158, 242, 355, 385, 400, 405],

and although their daily exposure to almost universal therapy is often cited as the cause [130, 170], this cannot be the whole explanation as Strept. pyogenes and Strept. pneumoniae have similar opportunities without comparable deterioration. It seems likely that a new factor, mutation-rate, must be taken into account in the choice of an antibiotic. Such a criterion would for example favour novobiocin [237] and vancomycin [501] of the newer drugs, as resistance to them is slow in onset, but would disqualify cephalosporin, micrococcin and albamycin to which resistance develops with unparalleled rapidity.

THE MECHANISM OF ACQUIRED RESISTANCE TO TUBERCULOSTATIC DRUGS

In the case of the drugs used for tuberculosis the very long period of treatment is especially conducive to the emergence of resistance. When streptomycin was first introduced and given alone, about one in four cases initially sensitive showed resistance towards the end of the second month of treatment [278, 300, 368], and this may well have accounted for many relapses [87]. Indeed it seemed only a matter of time before infections resistant from the start to streptomycin and other tuberculostatic drugs would be reported [82, 251].

Some reassurance from these forbodings was gained by the extensive trials conducted by the Medical Research Council [305]

using two or more drugs at the same time. As the mutations confering resistance to the different tuberculostatic drugs are quite distinct it was expected that the frequency of two or more such mechanisms occurring simultaneously in an individual bacillus would be of extreme rarity [211]. While in practice the onset of resistance in tuberculosis, as in other infections [133, 351, 410, 482], is certainly retarded by such devices, they do not appear to abolish the risk indefinitely [44, 434, 224], perhaps because the dose of one of the drugs, p-aminosalicylic acid (P.A.S.), is so formidable, or its deterioration to meta-amino phenolis such, that patients may fail to take what is prescribed [341, 433]. Doubly-resistant mutants should in theory arise at a frequency equalling the product of the single mutation rates, and if we knew these we could calculate the risk.

STREPTOMYCIN-RESISTANCE AS A SPONTANEOUS MUTATION

One must not exaggerate the dangers, particularly with in vitro tests which cannot comprehend the patient's own powers of recovery, and in other respects may not be strictly referable [29, 101, 267, 369]. For instance extreme streptomycin-resistance induced experimentally in cultures can lead to actual streptomycin-dependence as described in Chapter 8. The antibiotic, far from inhibiting the strain, encourages its growth [409, 492]. Such anomalies do not appear so far, however,

to have affected human cases, perhaps because they are readily reversible [334]. Synergism, the pronounced mutual benefit sometimes seen to occur among pairs of antibiotics[219,220,240,349,322], and its opposite - antagonism, do not seem to be demonstrable with tuberculostatic drugs [312], while the phenomenon of cross-resistance which occurs in other fields of chemotherapy [132] has not been a problem in the treatment of tuberculosis, except of course with analogues derived from the same parent drug. Moreover Linz and Lecocq [268] showed that in cultures from recovering tuberculous meningitis cases that streptomycin sensitive bacilli will outgrow resistant bacilli when the drug is discontinued: an example of the "Welsch Phenomenon" which is doubtless life-saving on occasions.

Paine and Finland [334] had shown experimentally some years earlier that both streptomycin-resistant and streptomycin-dependant clones actually grew slower than their sensitive counterparts, which may account for their relative rarity in nature. But they also showed [335] that these rare streptomycin-resistant and -dependant organisms could be recovered from strains of E. coli, Staph. aureus, and Proteus and Pseudomonas spp., given large enough inocula, and moreover that given the opportunity they would breed true. Working with Murray and Wilcox, Finland [323] had maintained thirteen strains of resistant Gram-negative bacilli through a hundred subcultures observing occasional sensitive colonies re-appearing

in two after 28 and 59 transfers. But it must be the steady ascendancy of such sensitive organisms by overgrowth from the start, rather than those only occasional back-mutations, to which we must look for their predominance in nature and presumably they possess a metabolic superiority, although Stubblefield [417] could detect no biochemical difference in streptomycin-resistant E. coli even when they also differed to a coccoid shape.

The advantage of using streptomycin-resistant mutants is that they demonstrate quite clearly that they owe their emergence and continued appearance entirely to the drug which is under our control. In fact we are dealing with selective breeding, and, as Klein [238] showed with resistant staphylococci, a high concentration of streptomycin is sufficient to disclose them, whereas successive more gradual concentration are needed with penicillin. In three subcultures he obtained Staph. albus 1250 times more streptomycin-resistant, and Price et al [348] in fourteen transfers raised the streptomycin-resistance of typhoid bacilli no less than 226,000 times. Later Goldstein [164] showed that a similar natural selection explained the emergence of streptomycin-dependant E. coli from rare mutants already present.

The irrefutable proof that streptomycin-resistant organisms pre-exist for the looking was obtained by the elegant

replica plates of the Lederbergs [261]. By making identical impressions of colonies on both streptomycin-containing and plain agar plates with a pad they showed conclusively that particular colonies were streptomycin-resistant though they had never been in contact with the drug until finally tested. Doubts raised by Stenderup [408] on the grounds that higher levels of streptomycin-resistance, e.g. 512 μg., only arose in his experience from initial resistances of from ½ to 2 μg., by successive cultures over a fortnight or a week respectively, are really further evidence of continued natural selection and not adaptation, as will be shown in Chapter 8. In fact Blondel [32] showed that by restricting the chance of mutations by deliberately keeping a Staphylococcal population below 10^5/ml., she was able to prevent streptomycin-resistance emerging altogether. Elek[117] tentatively suggested from early experiments that similar drug resistant mutants pre-existing in a typical E. coli population were likely to be of the order of 1 in 2 million for streptomycin, 1 in 5 million for chloramphenicol, and 1 in 15 million for oxytetracycline, which would explain the relatively greater frequency with which streptomycin resistance appeared clinically.

THE CONSTANCY OF STREPTOMYCIN-RESISTANCE MUTATIONS

From time to time there had been experiments suggesting that streptomycin-resistances of various degrees appeared with

a mathematical precision among multiplying bacteria. Thus Miller and Bohnhoff [314] in 1947 had shown that a strain of meningococcus had two such variants, one like the parent but resistant to from 1 to 10,000 μg., the other vary variable in appearance and actually dependant on 100 - 400 μg., of streptomycin. The following year Yegian and Vanderlinde [494] counted streptomycin-resistant colonies of Myco. tuberculosis H37Rv. by plating out liquid growths. Counts of the order of 1 per million were obtained resistant to 1 μg., with decreasing counts to 10 μg., and 100 μg., and they confirmed that the resistant to 1 μg., clone bred true. Moreover a strain of tubercle bacilli from a patient responding to streptomycin had similar counts, while a strain from a case relapsing after a month's treatment had a higher count of these resistant bacilli. This gave us prima facie evidence that streptomycin-resistant mutation rates would prove as predictable for Myco. tuberculosis as for other species.

CUMULATIVE AND PROGRESSIVE STREPTOMYCIN-RESISTANT MUTATIONS

Mitchison showed in 1950 [318] that the proportion of streptomycin-resistant among sensitive tubercle bacilli needed to give a resistant culture varied directly with the time available for incubation. Thus among his six strains proportions of 1 : 440 or even 1 : 180,000 could suffice given a fortnight, while rates of from 1 : 6 to 1 : 130 were

needed when only a week was available for their multiplication. This is of course, largely a reflection of the geometrical progression by which bacteria multiply. But not entirely; because, although on the average as little as 3% of resistant bacilli will overgrow a Dubos culture of tubercle bacilli in 10 days [317], there are considerable variations with the strain, and this is borne out clinically. For instance Mitchison [317] had among 18 cases of resistant tubercle bacilli, 10 which persisted six months, 8 of which rose to higher levels; but this very rarely reaches the extreme of streptomycin-dependance seen with E. coli and Pseudomonas sp. [495].

Demerec, who first showed the mutational origin of penicillin-resistant Staph. aureus in 1948 [92, 93, 118], also demonstrated what Bryson and Szybalski [425] termed the "Obligate Multi-step Pattern" of such resistance. By this was meant that the original strain had apparently only resistant mutants of low degree, - 4 per 100 million tolerating 0.12 units of penicillin in the case of Demerec's staphylococcus. With each subsequent subculture of the mutant clone in the presence of the drug, higher and higher resistance was built up. This is also the pattern with staphylococci and erythromycin or carbomycin [199, 203, 294], with Mycobacteria and neomycin [205], Esch. coli and chloramphenicol [62], and in fact with most bacteria and antibiotics.

With streptomycin-resistance in staphylococci, however, Demerec [93, 94, 118] was able to show a contrasting "Single-step Mutation Pattern". The very first culture isolated already contained resistant mutants of all degrees, and subsequent subculture continued to show a similar range. A like "Single-step Pattern" of streptomycin-resistance was shown for Esch. coli by Ushiba and Watanabe [454] and it is confirmed by the experiments described in Chapter 8. Ushiba's and others [454] and Tsukamura's team [448, 449] showed that Myco. tuberculosis behaved similarly in the presence of streptomycin, and this would explain the relatively abrupt onset of streptomycin resistance seen both clinically and by in vitro tests (Fig: 47).

There seems no reason to doubt that the more elaborate heredity mechanism described in the last chapter can also take part in drug-resistance such as that to streptomycin. When Griffiths [441] transferred genetic material between pneumococci his discovery concerned the capsular polysaccharide giving virulence; but it is now known that other inherited characters including drug-resistance can be so transformed, and Hotchkiss [202] conveyed streptomycin-resistance to a previously sensitive pneumococcus with desoxyribonucleic acid from a resistant strain in a matter of minutes in this way.

ISONIAZID

Resistance to isoniazid develops as rapidly as to streptomycin when the drug is given alone, in fact of the cases in the first British trial of the drug [303] 11% had resistant cultures after one month, 52% after two months and 71% after three. Middlebrook and Dressler [312] showed that the resistance to isoniazid developed in a single-step manner like that to streptomycin, but they confirmed that the two drugs were not synergistic [312], and Fisher [140] showed that haemin was required as a growth factor by the isoniazid-resistant bacilli, in fact without it the 'one-step' resistance did not occur. It is generally believed that isoniazid inhibits mycobacterial nutrition, though Pope [346] thought oxidation to be vulnerable. She points out that metabolic effects must bear no relation to loss of virulence. As mentioned later in Chapter 10 isoniazid-resistant mutants lose their pathogenicity for mice [228, 312, 320] and guinea-pigs [73, 311]; but they are still virulent unfortunately to higher animals including man [231, 252, 306], and it is clear that the mutations giving isoniazid-resistance must be very complex involving several genotypes.

PARA-AMINOSALICYLIC ACID

As regards P.A.S.-resistance Tsukamura [443] has suggested that there are two genotypes, one of low resistance

(1 - 2 μg./ml.) with a rate of about 1 in 50,000 and one high (5 - 100 μg./ml.) of a frequency of 1 in 100 million. Here we have some supporting evidence. In Slope Diffusion Tests as described in Chapter 10 this drug gives two zones of inhibition, one partial and the other complete. These are seen in Fig: 43, and enlarged in Fig: 44, where the more complex sensitivity pattern seen with P.A.S. in the right two tubes contrasts with the simpler inhibitory zones due to isoniazid in the three tubes on the left. Schmiedel's modification [384] of the Slope Diffusion Test has been used, that is to say long tubes instead of bottles cover the bigger inhibitory zones given by these very diffusible drugs.

There is another curious observation made by Tsukamura [447]. By studies of the size and number of surviving colonies of H37Rv strain of Myco. tuberculosis subjected to streptomycin and P.A.S., he was able to confirm what had been previously supposed, that the survival mutation rate to the two drugs was just the product of the survival mutation rates to each drug separately. Thus if the mutation rate to streptomycin is 1×10^{-m} and to P.A.S. 1×10^{-n}, that to the two is $1 \times 10^{-(m+n)}$. With isoniazid and P.A.S. however there is a complication [442]. P.A.S. - resistant strains are characteristically heterogeneous with bacilli of varying sensitivity and resistance present, and this heterogeneity of P.A.S.-resistance is still present in strains resistant to

both streptomycin and to P.A.S. But strains resistant to both isoniazid and P.A.S. are homogeneous, the bacilli being now all P.A.S.-resistant. If the isoniazid-resistant mutation removes heterogeneity, a characteristic of P.A.S-resistance, the two mutations must be in some way linked. Barreto [21] quotes Eagle as saying that such mutual linkage should make it very rare for the combined resistance to appear, and certainly when it does it is of high degree to both drugs. However that may be, P.A.S.-resistance seems no more suitable than isoniazid-resistance for simple experiments on mutation rates.

THE CHOICE OF STREPTOMYCIN-RESISTANCE AS A MEASURABLE MUTATION

Quite a number of substances can neutralise streptomycin [100, 139, 144, 264, 353, 366, 382], particularly reducing agents, and those bacteria which are naturally resistant such as Pseudomonas aeruginosa have been shown to have a protective enxyme 'streptomycinase' analogous to the penicillinase of naturally resistant staphylococci; but it does not seem to participate in mutant resistance any more than the latter. Unlike isoniazid streptomycin inhibits growth without gross morphological changes [245] and it is now agreed that the interference is with bacterial respiration[109,142,143] rather than protein metabolism [154, 175] as in the case of penicillin. All Mycobacteria are aerobes though tubercle

bacilli seem to be more tolerant than others [192] which may explain their ability to grow interstitially [97, 282].

The 1950 International Conference [271] showed that streptomycin reduced the oxygen consumption of sensitive organisms six times as much as resistant mutante, and there seems no reason to doubt that acquired streptomycin-resistance depends on some such compensatory respiratory mechanism whatever the species of bacteria, and that it is a relatively common mutation. We have therefore adopted it as the mutant for study in this thesis and have used strains of Esch. coli to observe the regularity of mutation rates while Myco. tuberculosis was more suitable for long-term epidemiological studies.

Tsukamura, observing cultures from 32 patients in Japan over 2 or 3 years, concluded that tubercle bacilli could achieve unlimited heights for streptomycin-resistance but not for resistance to P.A.S. or isoniazid [444]. Thus nearly half his cases reached a resistance to 1,000 μg./ml. streptomycin; but the upper limit for P.A.S. was 60 μg./ml. in about 60%, while for isonaizid there was a wide variation, 12% resisting 10 μg./ml. and 47% 1 μg./ml., while 41% stayed sensitive to 0.1 μg./ml. [446]. This could explain why streptomycin-resistance is relatively common and P.A.S.-resistance, at any rate at clinical levels, rare. We have

TABLE 4

DEGREE OF ISONIAZID-RESISTANCE

IN STRAINS OF MYCO. TUBERCULOSIS

20.11.61 - 19.11.62

Resistant to $> 5\ \mu g./ml.$	52	86.6%
Resistant to $> 1\ \mu g./ml.$		
but $< 5\ \mu g./ml.$	8	13.3%
Total	60	

not noticed such heterogeneity for isoniazid-resistance, in fact of 60 resistant strains tested during 1962 against isonaiazid 1 and 5 μg./ml., 52 were resistant to both and only 8 resistabt to 1 μg. only (Table 4).

Tsukamura also showed that the survival curves of populations of the H37Rv strain were discontinuous at two points, between 2 and 5 μg. streptomycin, and again between 20 and 50 μg., and he suggested that in Myco. tuberculosis separate genes controlled low and high resistance respectively[449]. As there was quite a variation in colony size among the lower strength mutants but uniformity among those at the higher strength he suggested a further distinction between the two genes, that the lower resistant one controlled other characters while the higher one did not [450]. But Ushiba has shown both in the case of Myco. tuberculosis and in Esch. coli that streptomycin-resistance probably depends on a number of genes [454]. The picture produced by the resulting cumulative mutation rates is the subject of our experiments in the following chapters.

CHAPTER 7. PREDICTABLE DRUG-FAST MUTATIONS AMONG PYOGENIC BACTERIA

TECHNIQUES FOR CALCULATING MUTATION RATES

PART A. EVERYDAY EVIDENCE OF DRUG-RESISTANT MUTATIONS

If bacterial mutations are so prolific why are they not seen more often in laboratory cultures? Routine drug-sensitivity tests with discs or tablets of antibiotic added to plate cultures have accustomed bacteriologists to clear-cut patterns of sensitivity and resistance. Not only are the zones of inhibition, which indicate a sensitive organism, usually sharply defined from the continuous growth elsewhere; but the zones themselves may be bounded by a luxuriant band of growth, where metabolism is apparently actually stimulated by the drug near its critical level. Sometimes this simulation is seen without the corresponding inhibition (e.g. round polymyxin in Fig: 19).

This failure to see mutants more often is not altogether surprising in view of their low incidence on any one plate, and the closeness of sensitivity of most to the parent strain. In clinical practice, where the tissue concentration of drug may only slightly exceed the minimum for general inhibition, their appearance will be much more favoured [423].

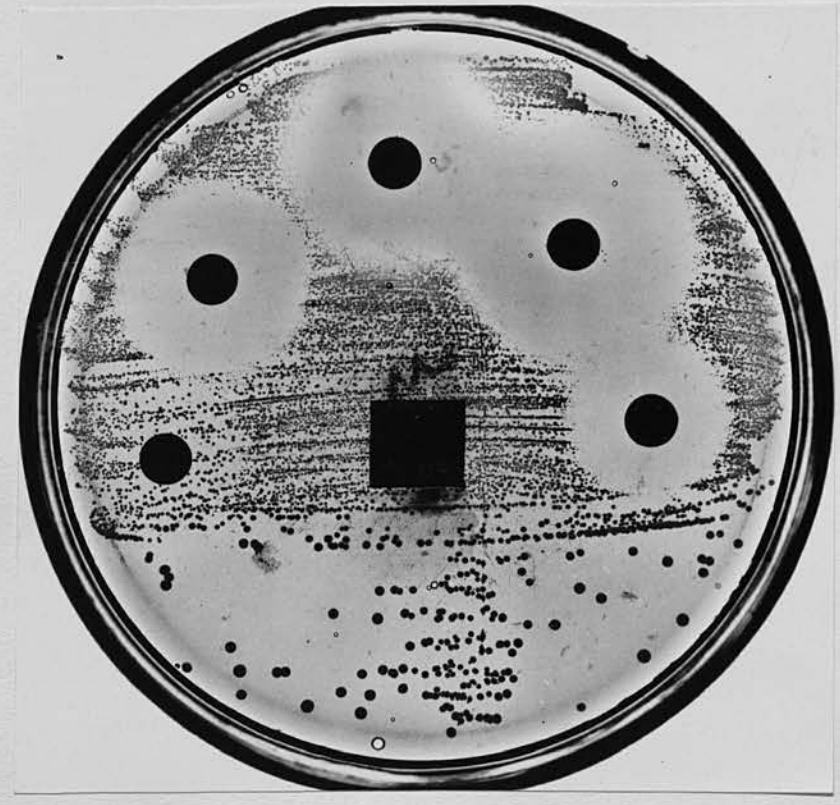


Fig: 13. *A Staphylococcal Drug-sensitivity Plate(No: 27858/60) showing complete resistance to sulphonamide (square patch) and many resistant mutants to oxytetracycline (first disc on left). Sensitivity is complete to the remaining discs, from left to right - streptomycin, penicillin, chloramphenicol, and erythromycin.*

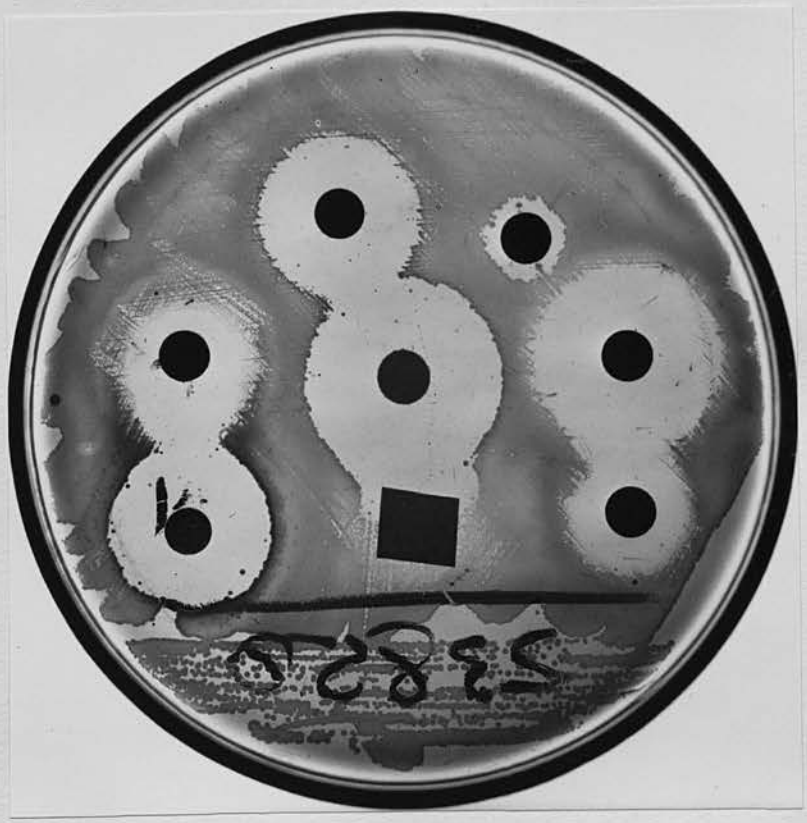


Fig: 14. *An example of synergism between framycetin (the central disc on the plate) and sulphonamide (the square patch). The latter has no inhibitory effect except on the side facing the framycetin disc, whose zone it 'draws out'. The other antibiotic discs are, reading clockwise:- oxytetracycline, streptomycin, penicillin chloramphenicol, and erythromycin. Strain Staph. aureus No. 23858/60.*

Sometimes, however, isolated colonies are quite conspicuous in the inhibitory zones, betraying the emergence of resistant variants, and a repetition of the test especially with a heavier inoculum [169], will show that it is a feature of that particular organism. Some strains are more prone to mutate than others, the tendency is a constant one, and only strains with a relatively high mutation rate are likely to show the phenomenon within the confines of a 4" Petri plate. Fig. 13 shows mutants resistant to oxytetracycline, Fig: 16 to chloramphenicol only, while in Fig: 18 mutants resistant to several antibiotics are seen.

In Fig: 14 growth of a strain of Staph. aureus is shown on an agar plate in actual size with zones of inhibition varying from ½ to 1" round 7 antibiotics (see legend). There is, in addition, a zone of inhibition adjoining the square patch of sulphonamide; but only on the side facing framycetin - an instance of synergism between these two drugs.

Fig: 15 shows another strain of Staph. aureus sensitive only to chloramphenicol and eythromycin. The two larger colonies in the inhibitory zone round erythromycin and the few similar large colonies scattered round chloramphenicol are coliform contaminants. The most striking feature, however, is that the areas of no growth are abrupt, except round chloramphenicol where three distinct zones occur -

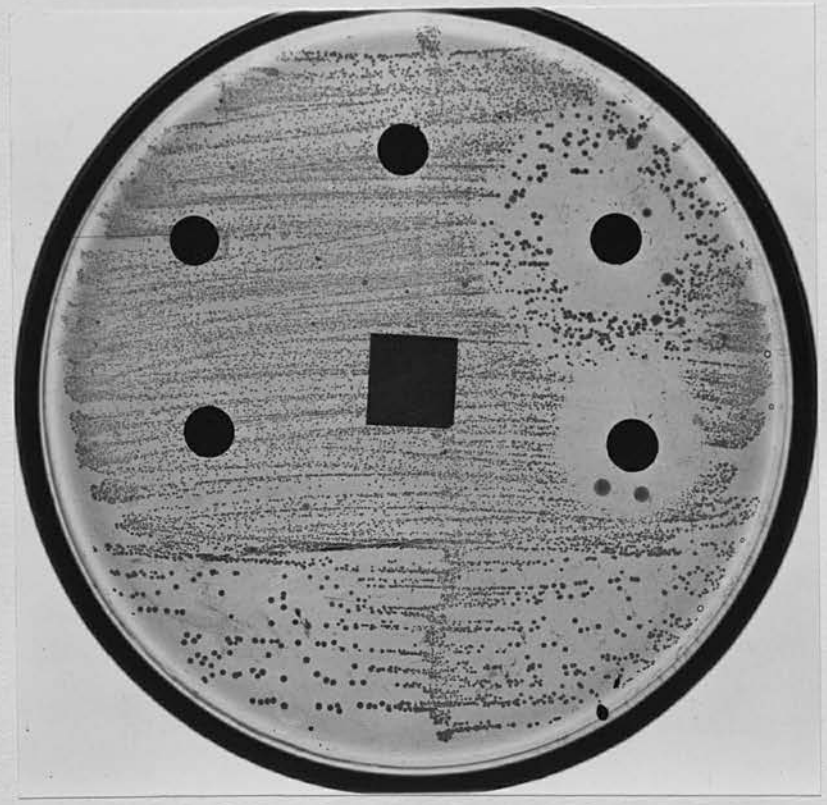


Fig: 15. *A drug-sensitivity test on a strain of Staph. aureus, No. 27074. Reading clockwise the antibiotic discs are oxytetracycline, streptomycin, penicillin, chloramphenicol, and erythromycin, with sulphonamide in the square patch. The Staphylococcus, characteristic of a hospital infection, is inhibited only by the chloramphenicol and erythromycin discs (the occasional larger colonies are a coliform Note, however, that while the erythromycin zone is clear-cut, round the chloramphenicol there is an intermediate zone, which is generally considered to represent a bacteriostatic level of drug giving only partial inhibition. Unlike the growth seen with resistant mutants, it occurs in a band which is itself sharply defined from full growth on the one side and no growth on the other.*

Fig: 16. *Drug-sensitivity test on Staph aureus strain No. 4161/61 from a skin lesion in a man of 21. The antibiotic discs here are, reading clockwise, oxytetracycline, streptomycin, penicillin, chloramphenicol, and erythromycin, with sulphonamide in the square patch. The organism is a typically resistant hospital strain of Staph. aureus. Only erythromycin and chloramphenicol are inhibitory, and the zones of both, but especially chloramphenicol, contain resistant mutant colonies scattered haphazardly within the 'inhibitory zones'.*

complete clearing, partial growth, and full growth. The middle zone in such a case is generally agreed to indicate a bacteriostatic level of the antibiotic contrasting with the bactericidal zone of full inhibition. Contrast this with Fig: 16 where a strain of Staph. aureus, again showing sensitivity only to chloramphenicol and erythromycin, has a very small proportion of the colonies persisting throughout these inhibitory zones and in the apparently haphazard manner characteristic of true mutants. Fig: 17 shows that the chloramphenicol-resistance, for example, of these is an isolated phenomenon, because, when tested in parallel to a dozen other antibiotics, the mutants in all other respects resemble their parent strain.

Sometimes, however, a strain shows mutants resistant to a number of antibiotics, and Fig: 18 is an example of this in a Staphylococcus from a chronic varicose ulcer. Many years' intermittent treatment has produced a strain wholly insensitive to oxytetracycline and sulphonamide, while currently mutants are appearing resistant to streptomycin, penicillin, chloramphenicol and erythromycin. Matching sensitivity tests in Figs: 19 and 20 compare the subsequent behaviour of the streptomycin-resistant mutant with the parent growth. In Fig: 19, after overnight incubation, they are identical except that the former has mutants also against kanamycin. In Fig: 20 a further day's incubation has produced

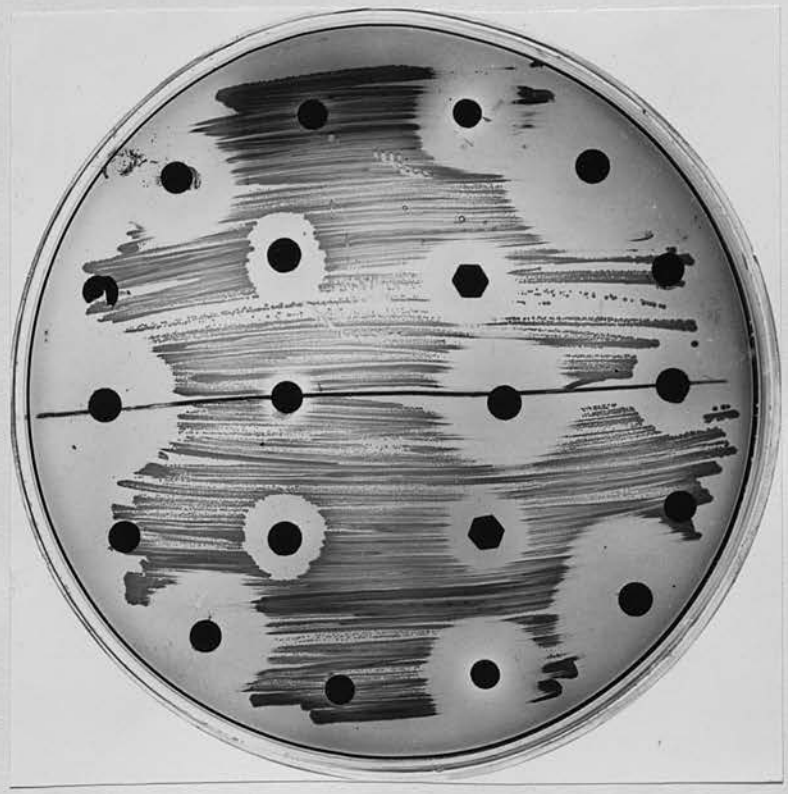


Fig: 17. *A drug-sensitivity test in parallel with the parent strain of the hospital Staph. aureus from the culture seen in Fig: 16, and its chloramphenicol-resistant mutant (below) shows that both are identical in response to twelve other antibiotics:-*

Framycetin	*Bacitracin*	*Oleandomycin*	*Ristocetin*
Streptomycin	*Vancomycin*	*Spiramycin*	*Oxytetracycline*
		hexagonal patch	
Chloramphenicol	*Polymyxin*	*Novobiocin*	*Neomycin*

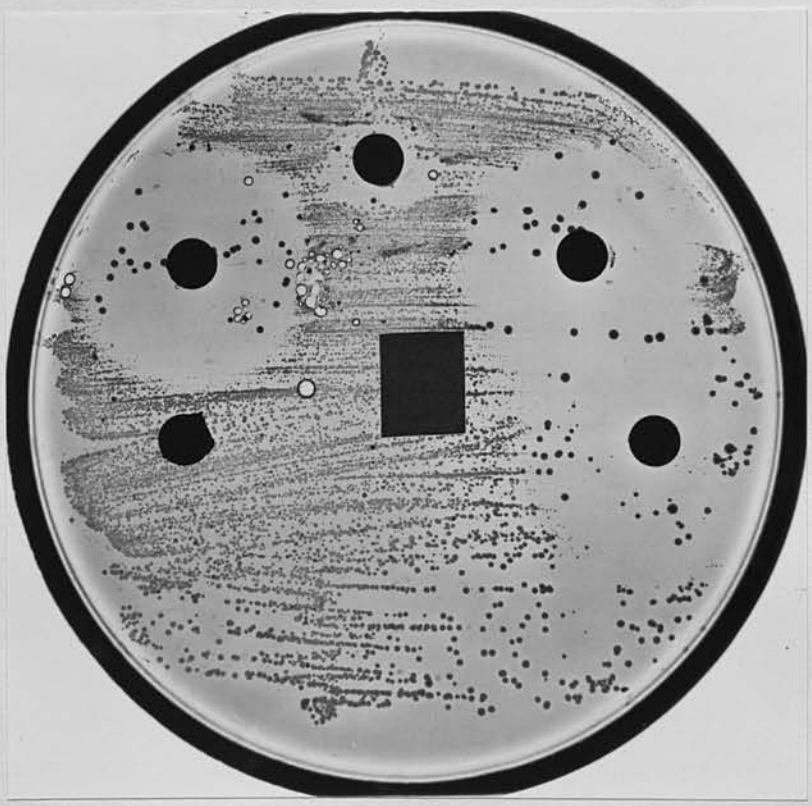


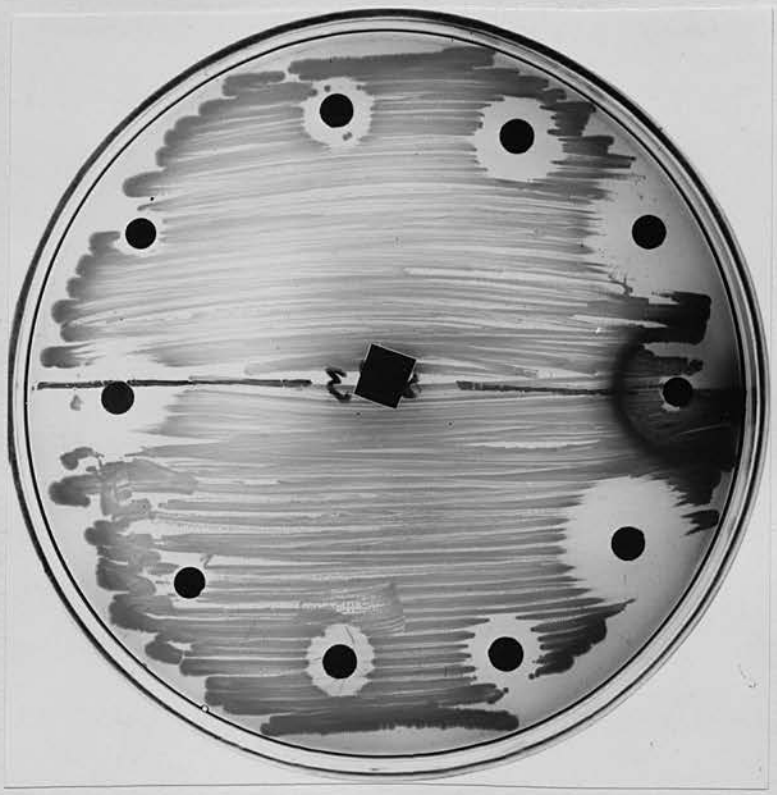
Fig: 18. *A culture (23883/60, Elizabeth Mungall) of a 20-year old varicose ulcer showing a strain of Staph. aureus with multiple resistant mutants. The drugs and their layout are the same as in Fig: 16. It will be seen that in this case none are completely effective. Probably as a result of years of intermittent treatment there is now total insensitivity to oxytetracycline (extreme left) and sulphonamides (square patch), while mutants are widespread in the zones round all the other drugs.*

a crop of mutants also against neomycin in the streptomycin-resistant variant. Kanamycin and neomycin are known to exhibit some cross-resistance with streptomycin [415], and this would seem to be evidence of this.

MULTIPLE INFECTIONS BY DIFFERENT BACTERIA

An incidental use of drug sensitivity tests is to distinguish the various genera and species of organisms. Thus penicillin and erythromycin have virtually no effect on coliform bacilli, so that if a zone of partial inhibition is seen in a urinary culture there is evidence of some co-existing sensitive organisms such as a Gram-positive coccus. The reverse picture is seen in sputum cultures, for if Neisseria catarrhalis or Haemophilus influenzae are present, their existence will be most conspicuous where they are growing without competition from the pneumococcus, round penicillin for H. influenzae, and round both penicillin and erythromycin for the Neisseria. In pus haemolytic streptococci can be outgrown by staphylococci; but the much greater sensitivity of the latter to neomycin and framycetin enables Strep. pyogenes to be distinguished inside the inhibitory zones round these antibiotics.

In view of the frequency of such simultaneous infection by two or more species of bacteria, there is no inherent



Figs: 19 and 20. *Demonstrate probable cross-resistance between streptomycin, kanamycin and neomycin.*

Fig: 19 *Additional drug-sensitivity tests performed in parallel on the parent strain of Staph. aureus seen in Fig: 18 (upper half of plate), and on a representative sweep of its streptomycin-resistant mutants (lower half of plate) show that they are not also otherwise identical. Reading clockwise (upper half of plate), framycetin is effective, spiramycin only very weakly so, kanamycin shows three mutants, novobiocin and neomycin good zones, polymyxin no effect.*

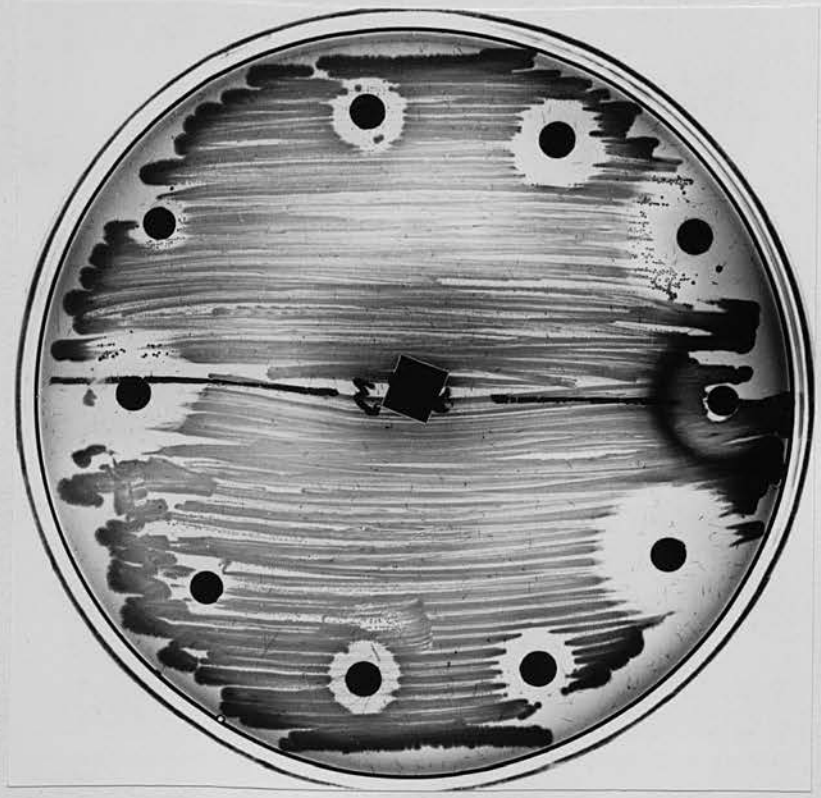


Fig: 20. *The additional drug-test in Fig: 19 was read and photographed after overnight incubation. But here we see the same plate after a yet further day's incubation. It will be noted that, in contrast to the parent strain (lower half of plate) the representative resistant mutant strain (upper half) which already differed in having three colonies insensitive to kanamycin, has now also late resistant mutants to neomycin. Both these antibiotics are known to exhibit cross-resistance with the streptomycin already seen to be ineffective.*

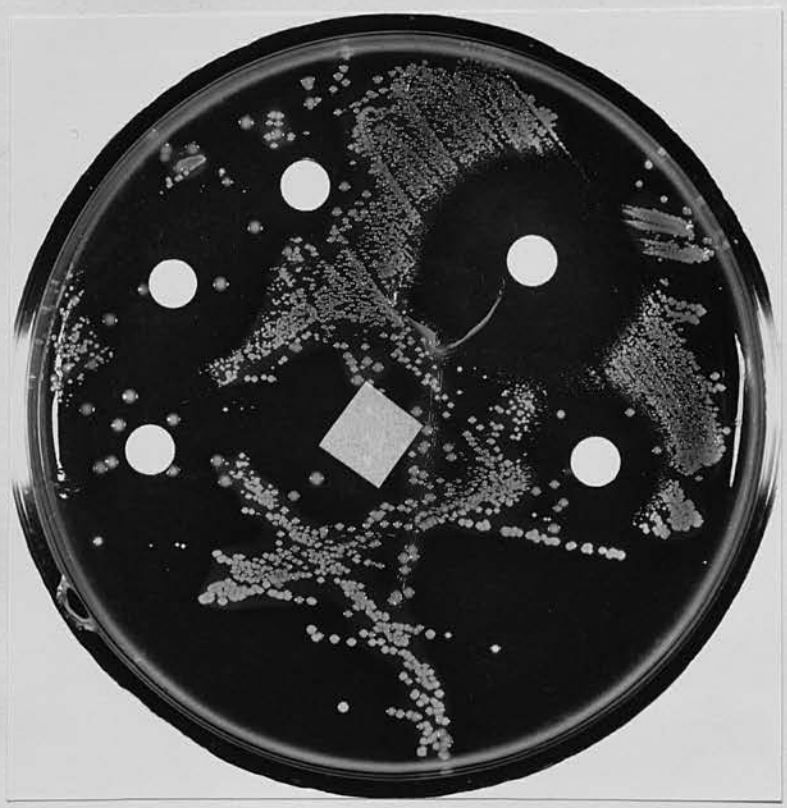


Fig: 21. *A Double Staphylococcal Infection with Mutants.*

Fig: 21 shows the complex picture produced by two strains of Staph. aureus with differing drug - sensitivity patterns, the inhibitory zones being invaded by resistant mutants derived from each. It will be noticed that one strain, represented by hollow circles labelled 'a' in the key, Fig: 22, has paler and more discrete colonies than 'b' represented by solid circles, and 'phage typing differentiated the two as (a) 52A+ and (b) 6/47/53/54/81w. The two strains had produced an Otitis externa in a woman aged 61 years.

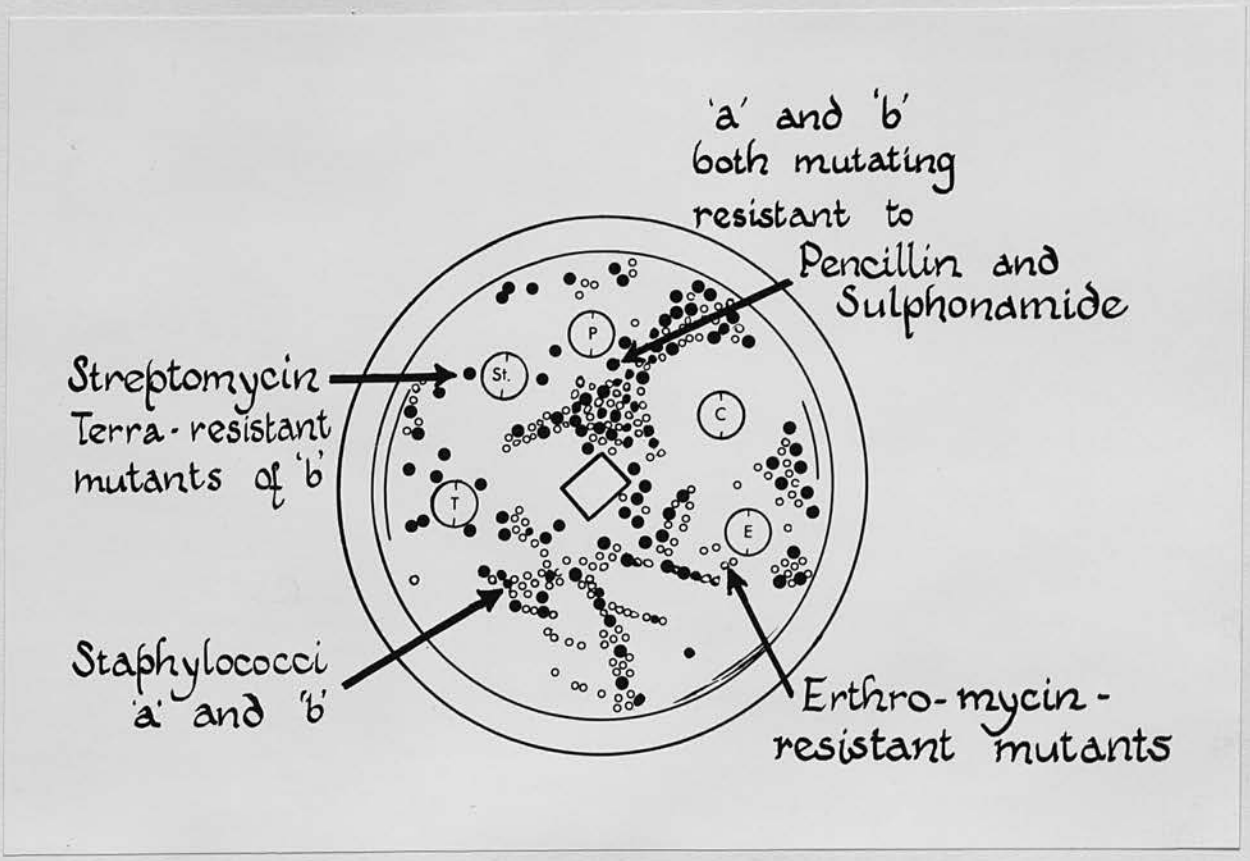


Fig: 22. *Diagramatic explanation of the photograph in Fig: 21.*

P = penicillin, St. = streptomycin, C = chloramphenicol,

T = oxytetracycline, E = erythromycin,

square patch = sulphonamide.

improbability of similar mixed infections with different strains within a species. Hitherto these would generally have passed unnoticed or been regarded as mere variants in colour or other naked - eye characters; but antibiotic sensitivity tests give, in effect, a second biological classification superimposed on the traditional one of colonial morphology. The very complicated picture which can ensue when there are two strains of one species which, in addition, are both mutating is seen in Figs: 21 and 22.

A double Staphylococcal infection which presented almost chimerical features on culture is illustrated in Fig: 23. The main growth of Staph. aureus is sensitive to streptomycin; but within the inhibitory zone, and adjoining the antibiotic tablet '3', is a paler growth of Staph. albus, which is also Coagulase-positive. Clearly the albus strain is drug-resistant; but what remains to be explained is the intervening clear zone between the two. A subculture growing without streptomycin excludes streptomycin - dependance, which would be the simplest explanation, and Waterworth has shown that such rare anomalies are due to staphylococcine production by the predominant growth masking the subordinate culture, except where it lies protected near the antibiotic. The antagoniam between the two strains is shown by superimposing subcultures (Fig: 23 inset).

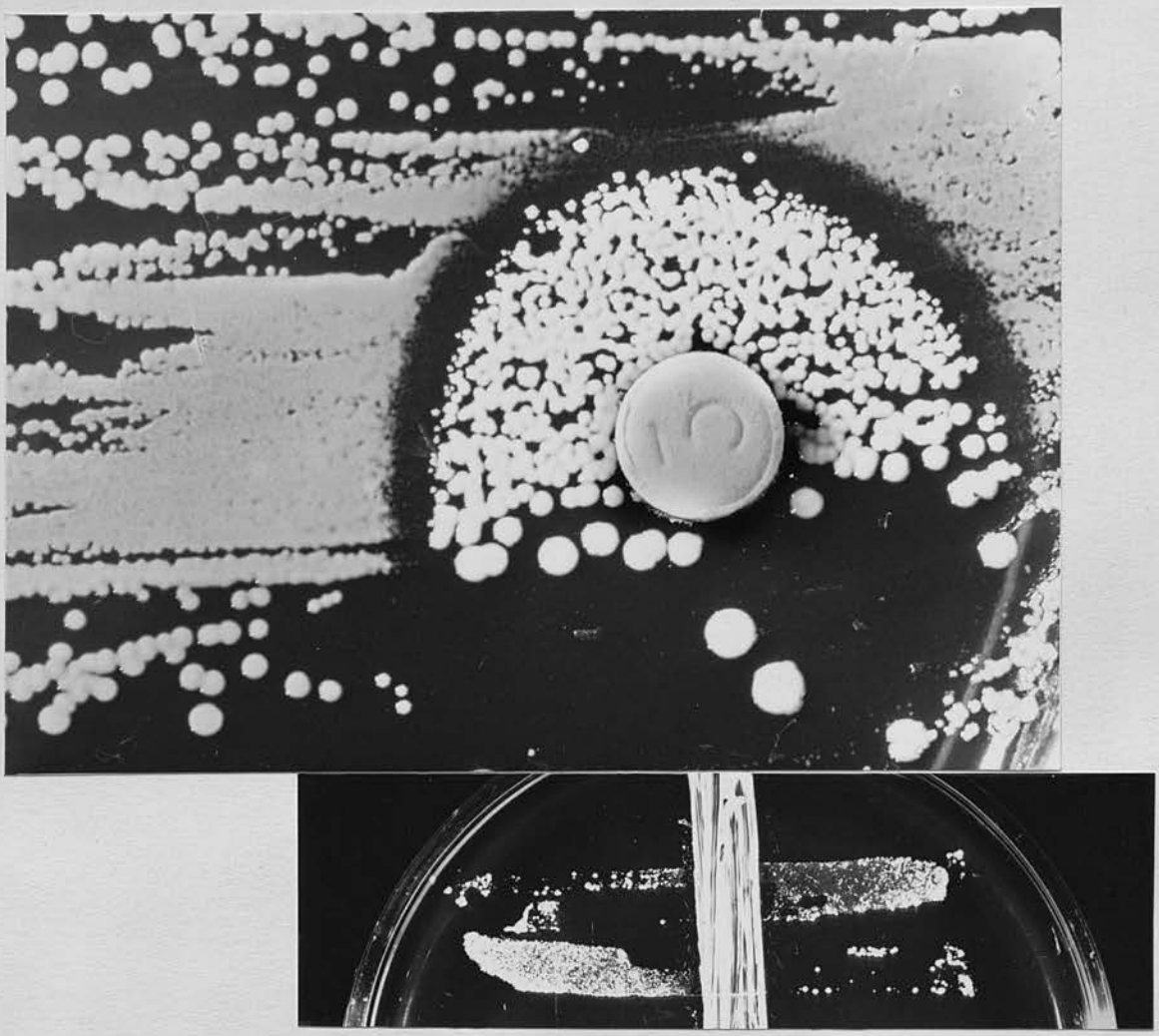


Fig: 23. *A curious anomaly. Two mixed strains of Staph. aureus have been revealed by streptomycin:- (a) streptomycin-sensitive (b) streptomycin-resistant. The zone between the two is explained by the fact that (a) is producing a staphylococcine against (b), which, in fact, is only able to grow when (a) is not present. This is proved by a streak plate with the two strains showing their growth incompatibility quite apart from the intervention of streptomycin. (Insert).*

ANTIBIOTIC DIFFUSION GRADIENTS

The inhibitory zones round the drugs in the sensitivity-tests which we have so far described may be regarded as miniature gradient plates on which, as described by Szybalski [423], variants have the opportunity to appear in isolation, freed from all competitors except those sharing their degree of resistance, those nearest the diffusing antibiotic having to withstand the highest concentrations and so on in proportion to their distance outwards. In Chapters 10 and 11 a technique, Slope Diffusion, is described for studying the mutants with different degrees of resistance arising in strains of Myco. tuberculosis. Ordinarily those very scanty mutants are seen which are of relatively high resistance, and they appear as discrete colonies on the drug-incorporated slopes of the solid culture sensitivity-tests (Figs: 39 and 40). Cutter-plates are unlikely to provide enough clear zones (Fig: 41) and sensitivity-tests in liquid medium will not show the frequency of mutants at all.

THE CONSTANCY OF BACTERIAL MUTATIONS

If a pure culture of a bacterium such as Escherichia coli is spread equally on plates of media with and without known amounts of antibiotic, the drug-containing plates will grow only isolated colonies whose number, even though small, is remarkably regular. In Fig: 24 is seen a comparison of

Fig: 24. *The in vitro effect of streptomycin. The left-hand plate contains plain MacConkey Agar, the right-hand one similar medium incorporating 5 μg./ml. streptomycin at the time of pouring. The plates were each uniformly spread with the same inoculum, 0.1 ml. of a broth culture of Escherichia coli N.T.C. 1094 diluted 10^5. Over 2,000 colonies as shown by the left-hand plate are reduced to 1 by the 5 μg./ml. streptomycin in the right.*

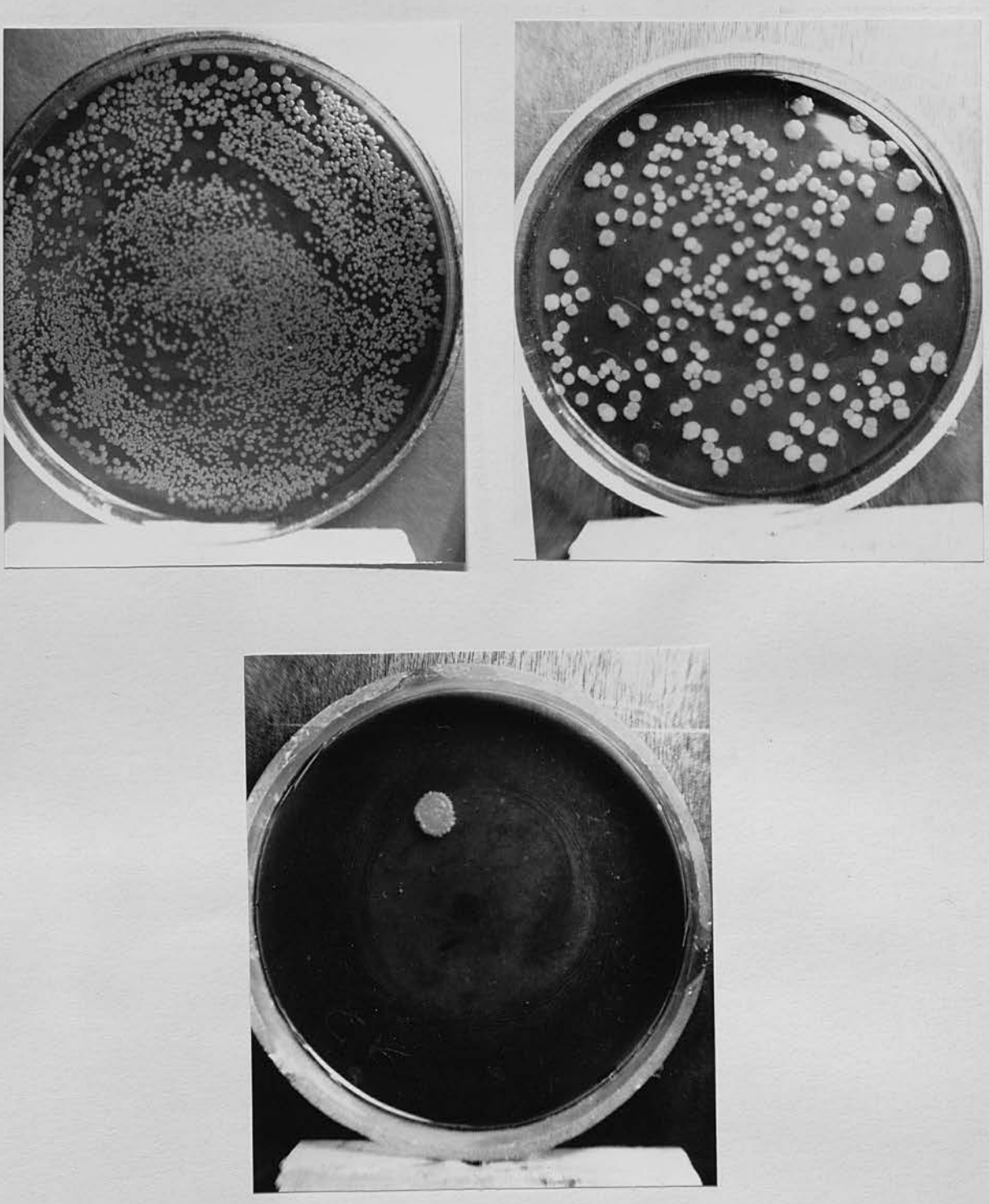


Fig: 25. *The proportionate effect of incorporating increasing amounts of streptomycin in MacConkey Agar on the growth of a strain of E. coli neapolitana. Over 3,000 colonies on the normal plate 'A' are reduced to 235 by 1 µg./ml. in 'B', and to 1 colony by 2 µg./ml. in 'C'.*

colony counts of E. coli National Type Culture No: 1094 on MacConkey Agar and on similar medium containing 5 μg/ml. of streptomycin. The single conspicuous colony on the latter is derived from the sole survivor of some 2000 bacilli placed on the plate, and the experiment can be repeated again and again with impressively consistent results.

Moreover if a series of plates are prepared with various known amounts of the antibiotic the mutants will fall off in numbers in direct proportion to the strength of the drug, the numbers surviving at each concentration being remarkably constant. In Fig: 25 are shown the numbers of a laboratory isolated strain of E. coli neapolitana withstanding 1 and 2 μg/ml. streptomycin respectively.

PART B. A TECHNIQUE FOR CALCULATING MUTATION RATES

Principle

It seemed clear that, if a reliable quantitative method of estimating the proportion of these mutants could be devised, then the mutation rates at various drug levels could be determined for strains of any species of organism. Experiments will be described showing that such rates are constant for individual strains, and that drug-resistant mutant clones will mutate in turn at constant rates so that, where a drug is constantly applied, progressive mutations

will occur at mathematically predictable rates. The actual time of their emergence will, of course, be the product of these mutation rates and the generation time which is also a constant for any individual organism in the log phase as we shall show. Expressed mathematically this can be summarised:-

Time of Emergence = Mutation Rate x Multiplication Rate,

and there will theoretically be an equation such as this for each degree of antibiotic resistance, all acting cumulatively and in unison. Natural selection by the continuing action of the antibiotic will then ensure that the resulting galaxy of mutants replaces entirely the original sensitive 'population',

Serial Decimal Dilutions

The essential requirement for accurate plate counts of bacteria is a reliable method giving known dilutions of the inoculum. Collecting too few colonies precludes any reasonable assessment of the actual numbers of mutants present in nature, while too many immediately introduces counting errors. But besides this Elek [118] has pointed out that a heavy inoculum will actually use up the antibiotic, and he quotes calculations that, in the case of the Staphylococcus and penicillin, each coccus binds 750 molecules of the drug [110, 111, 274, 275].

EXPERIMENT 1.

PRELIMINARY STUDIES OF RELIABILITY OF DILUTIONS

The test organism was a laboratory-isolated strain of Escherichia coli var. neapolitana (dulcitol-fermenting + and motile). This was grown overnight in nutrient broth [284] incubated at 37°C. in two cotton-wool-plugged tubes. The resulting cultures were checked for comparable growth, and complete dispersion ensured by two hours' mechanical shaking in a Kahn shaker with two glass beads in a bijou screw-capped bottle. Poured back into a single $\frac{5}{8}$" diameter test-tube, the turbidity was adjusted to Brown's Opacity Tube No: 4 (approximately 3,000 million bacilli per ml.) by sampling and adding sterile normal saline by pipette in appropriate amounts.

Serial tenfold dilutions of this standardised inoculum were now made down to 1/million by setting up a rack of $\frac{5}{8}$" sterile test-tubes each containing 9 ml. sterile saline, adding 1 ml. of standarised culture to the first with a 10 ml. pipette and thoroughly mixing by twenty rapid withdrawls and expulsions. This was repeated six times down the line of tubes using five separate 1 ml. pipettes for the subsequent transfers and mixing.

Statistical analysis [399] shows that pipetting errors are small provided a fresh oven-sterilised 1 ml. pipette is

used at each stage to avoid carrying over undiluted inoculum on the outside surface of the glass or possibly from the upper bore. Experience soon showed that this use of dry-sterilised pipettes was preferable to those silicone-treated for the same purpose, and the same 1 ml. pipettes can remain upright each in its own test-tube of diluted culture for the subsequent inoculation of the plates.

Snyder [399] showed that 0.1 m. spread with a glass rod gave reliable counts of dysentary bacilli, and Novel [330] counting bacteria in sea water showed that 2 plates were useless for significance, 10 plates were excellent and 5 almost as good. Therefore well dried nutrient agar [284] was used in 15 4" Petri plates, and 0.1 ml. of the 1/million dilution was placed in the centre of each of 5 plates, 0.1 ml. of 1/100,000 dilution on each of the next 5 plates, and 0.1 ml. of 1/10,000 dilutions on the last 5. The inocula were then rapidly spread by revolving the plates under an angled glass-ros previously sterilised by flaming and then cooling. There is no need to repeatedly resterilise the rod throughout the procedure provided the spreading is done progressively from the weakest concentration upwards and is lightly flamed after each 5 plates, i.e. on actually changing inoculum strength. Any carry-over within each group of 5 plates will be compensated by subsequent averaging.

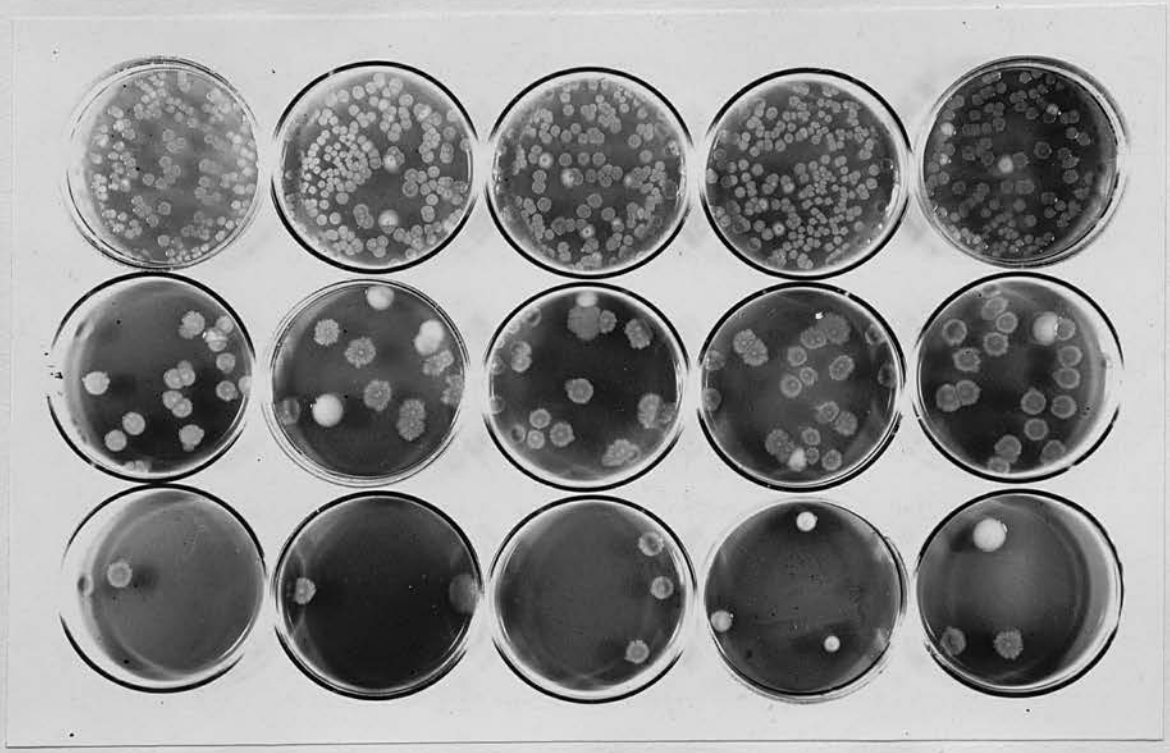


Fig: 26. *An experiment showing that 10-fold dilutions are an effective technique for making measurable counts if fresh pipettes are used at each step:-*

5 plates averaging 200 colonies each in the top row are reduced by ten-fold dilution to 5 averaging 20 in the middle row, and by further ten-fold dilution to 5 averaging 2 colonies each in the bottom row.

The results of this method of serial decimal dilutions are shown in Fig: 26. It will be seen that the 5 upper plates average approximately 200 colonies, the next 5 average 19, and the last 5 average 2.4. Ideally, it might be supposed, one would want to count the maximum number; but it will be clear from Fig: 20 that it is not feasible to use more congested plates than the middle set. In practice it is best to aim at counts of about 20 colonies, and this has meant prefacing all the experiments which follow with a pilot experiment in each case as a guide to the choice of dilutions subsequently.

EXPERIMENT 2.

PREDICTABLE RESISTANT MUTATIONS IN A STRAIN OF E.COLI VAR. NEAPOLITANA

Materials

The same laboratory-isolated strain of E. coli var. neapolitana was used as in the previous experiment, as that experiment could be taken as a guide for the inoculum. Moreover its discrete colonies had been easy to count, and such separation of colonies varies considerably between species and even strains. The tubercle bacillus is so cohesive that it is extremely difficult to keep individuals apart, and Klebsiella confuses by the tendency of its colonies to coalesce.

Streptomycin was chosen as the antibiotic. It is effective on both the genera Escherichia and Mycobacteria which we wished to study as examples of fast and slow growing organisms. Its mode of action is generally believed to be an interference with the enzyme controlling the final stage of bacterial respiration [142, 143], possibly by the oxidation of benzoic acid [109] or some amino-acid [69, 154, 175]. Presumably at least one alternative respiratory enzyme system is available, and this could explain the relatively high mutability of many bacteria, especially these two genera, to this drug.

MacConkey's bile-salt neutral red lactose agar was used as medium [284]. It is true that the pH of the medium is important, and that at 7.6 the alkalinity increases streptomycin's potency twelve times [1, 110, 348]. As against this peptone in the medium antagonises the antibiotic [264, 365] though far less actively. Fortunately we are not so much concerned with the absolute numbers of surviving bacilli but with their ratio to the total bacterial population, so that with the proviso that our final drug strengths are nominal, we can make deductions about high and low strength mutations.

Method

The test strain of Escherichia coli was grown overnight in nutrient broth at 37°C, dispersed by agitation as before, and standardised by turbidity again to approximately 3,000 million bacilli per ml. The resulting suspension was again found to be adequate, and shaking was therefore adopted for all subsequent experiments in preference to using emulsifying agents such as sodium mono-oleate ("Tween 80") [105] which can reputedly exaggerate the effects of streptomycin as much as a thousand times [116, 139] giving the risk of a wholly disproportionate result, even if the bacilli are partly protected by adding protective bovine albumin [103, 382].

A 250-ml. bottle of MacConkey agar was melted and sterilised in the autoclave, and allowed to cool to 60°C.

TABLE 5

FREQUENCY OF RESISTANT INDIVIDUALS

IN VARIOUS STRAINS OF *ESCHERICHIA COLI*

E. coli var. neapolitana, Laboratory source (dulcitol +, motile)

Conc. of Streptomycin in µg/ml.	Diln: of Inoculum	Colony counts at 3 days				Average	Rate per 100 million
0	10^6	98	105	99	104	101.5	100 million
1/8	10^6	77	130	109	99	103.75	102.2 million
1/4	10^6	109	107	105	102	105.75	104.2 million
1/2	10^6	91	98	97	83	92.25	90.88 million
1	10^6	11	13	12	12	12	11.82 million
2	10^4	2	5	3	4	3.5	34,490
5	10	2	3	2	2	2.25	2,217
10	1	0	0	0	0	0	0

Streptomycin hydrochloride solution, freshly prepared in its vial, was added to the plates at the time of pouring, each plate getting 5 ml. of medium and streptomycin to give final concentrations of 0, $\frac{1}{8}$, $\frac{1}{4}$, $\frac{1}{2}$, 1, 2, 5 and 10 μg/ml. Four plates were prepared of each strength.

0.1 ml. amounts of the appropriately diluted inoculum were pipetted on to each plate, the 1/million dilution being used for the 1 μg/ml. plates and all weaker concentrations while 1/10,000, 1/10 and undiluted inocula were used for the 2, 5, and 10 μg/ml. concentrations respectively. Well-dried plates were used as in the previous experiment, and quick spreading ensured by revolving the plates under a sterile angled glass-rod as already described. The 32 plates, 4 of each strength of antibiotic, were incubated for three days at 37°C with the results shown in Table 5.

It will be seen that no effect appears on the two lowest concentrations of streptomycin compared with '0' (the control), and very little from $\frac{1}{2}$ μg/ml. About 10% of the strain can survive 1 μg/ml., and 1 in 3,000 bacilli survive 2 μg/ml., but higher doses produce a sharp diminution in the count to 1 in 50 million at 5 μg/ml., and a complete inhibition with 10 μg/ml.

TABLE 6.

FREQUENCY OF RESISTANT INDIVIDUALS

IN VARIOUS STRAINS OF ESCHERICHIA COLI.

E. coli var. neapolitana Laboratory source (dulcitol +, motile).

Confirmatory experiments gave respectively:-	*Resistant to 1 μg/ml. - (a) 10.9 (b) 10.76 (c) 12.01 millions per 100 million.*
	Resistant to 2 μg/ml. - (a) 32,360 (b) 33,900 (c) 44,740/100 million
	Resistant to 5 μg/ml. - (a) 1.69 (b) 2.58 (c) 2.66/100 million.
	Resistant to 10 μg/ml. - (a) 0 (b) 0 (c) 0/100 million.

But even more significant than the precise ratios is the mathematical certainty with which each level of mutation rate is regularly reproduced. The whole of Experiment 2 was now repeated three times with no change in any detail, and the four sets of mutation rates, each set calculated as in Table 5, are summarised in Table 6. It will be seen that in spite of variations of some 10% in each rate, the mutation rates are in fact quite distinct and, within the limits of experimental error, reproducible apparently indefinitely and therefore predictable.

CUMULATIVELY PROGRESSIVE MUTATIONS

Regularly repeating mutations of this sort must account for the gathering accumulation of mutant bacteria from which overall resistance can be confidently expected in a certain time. For example in the days of single-drug therapy of tuberculosis a quarter of the patients had streptomycin-resistant strains by the end of the third month[138,278]. What now remains to be explained is the abruptness of this transition. Although patterns of resistance vary from the single-step of streptomycin through intermediate types to the multi-step of penicillin [445], these differences are only matters of degree. The essence of mutational change is its suddenness.

If mutations continued in series at precisely the same rate a relatively gradual change would occur. But the mutants are themselves mutating, for as Burnet [50] says: "growth, reproduction and mutation are the fundamental characteristics of life". If we can show that the mutants in turn mutate at fixed rates we will have a possible mechanism, a mobilis in mobile, whereby myriads of more or less successful individuals will all emerge together to give the explosive outbreak so typical of the recurring epidemic.

In the next chapter detailed experiments with progressive mutations will be described; but a simple test with our present culture will show that resistant variants not only maintain their resistance but can yield still more resistant progeny in their turn.

EXPERIMENT 3.

"STREAK-PLATES" AS EVIDENCE OF BACTERIAL VARIANTS SHOWING FURTHER MUTATION

Materials and Method

A series of drug-resistant clones were obtained by picking off isolated mutant colonies, one from each of the various antibiotic-containing plates, and one from the control plate, of Experiment 2 and similar experiments. Each was grown overnight in a tube of nutrient broth at 37°C, the tubes being labelled with the reputed degree of resistance in micro-grammes. The resulting cultures were adjusted with broth to a uniform turbitity (Brown No: 4 - 3,000 million E. coli per ml.).

Single uniform loopfuls, yielding approximately 0.01 ml. of each broth culture, were streaked in parallel on each of a duplicated series of MacConkey agar plates to which streptomycin hydrochloride had been added at the time of pouring to give final strengths of 0, 1, 10 and 100 μg/ml.

Results

The eight plates, 2 each of 4 concentrations of drug, were incubated at 37°C for 6 days to ensure the appearance of even slow-growing mutants. The results are recorded in Table 7.

TABLE 7

"STREAK-PLATE" EVIDENCE OF DRUG-RESISTANT MUTATIONS

Clones of E. coli, N. T. C. 1094, derived from the previous experiments were inoculated in parallel on fresh drug-containing plates with the following results after overnight incubation at 37°C :-

	MacConkey Agar Plates incorporating Streptomycin.			
	Control - no drug.	*1 μg./ml.*	*10 μg./ml.*	*100 μg./ml.*
Nominally Sensitive	∞	∞	2	0
" *Resistant to 1 μg.*	∞	∞	6	0
" " *10 μg.*	∞	∞	∞	0
" " *100 μg.*	∞	∞	∞	∞

It will be seen that the degree of overall resistance in each streak is at least as high as in its parent inoculum, so that the clones are to that extent "breeding true". But in addition the nominally "Sensitive", and "Resistant to 1 μg/ml." clones are each yielding a few colonies also resistant to 10 μg/ml.

DRUG-RESISTANT MUTATIONS IN A TYPE STRAIN OF *E. COLI COMMUNIS*

The possibility remained that such regular and progressive mutagenicity was exceptional if not peculiar to the particular strain of *E. coli neapolitana* chosen. A similar simple "streak plate" experiment was therefore performed with a type culture of *E. coli communis*.

EXPERIMENT 4.

Materials and Method

Drug-resistant clones of various degrees were obtained as follows. Streptomycin hydrochloride was added to MacConkey's agar medium at the time of pouring to give plates with concentrations of 0, 1, 5, 10, 50 and 100 μg/ml. respectively. Large inocula of the order of 30 million E. coli communis, N.T.C. No. 1094, were placed on each plate as loaded loopfuls of an overnight broth culture. The inocula were spread by a succession of parallel strokes of the loop repeated three times at right angles round the periphery of the plate.

From each plate a single isolated colony was picked off from the surviving resistant mutants. This was cultured in turn by overnight incubation at 37°C in nutrient broth. The set of six broth cultures, each representing a distinct clone, were all adjusted to uniform turbidity (Brown No. 4 - 3,000 million E. coli per ml.) and loopfuls of each, approximately 0.01 ml., were streaked in parallel on a series of antibiotic-containing plates. These again were of MacConkey's agar medium with 5, 10, 50 and 100 μg/ml. streptomycin added as solution of the hydrochloride at the time of pouring.

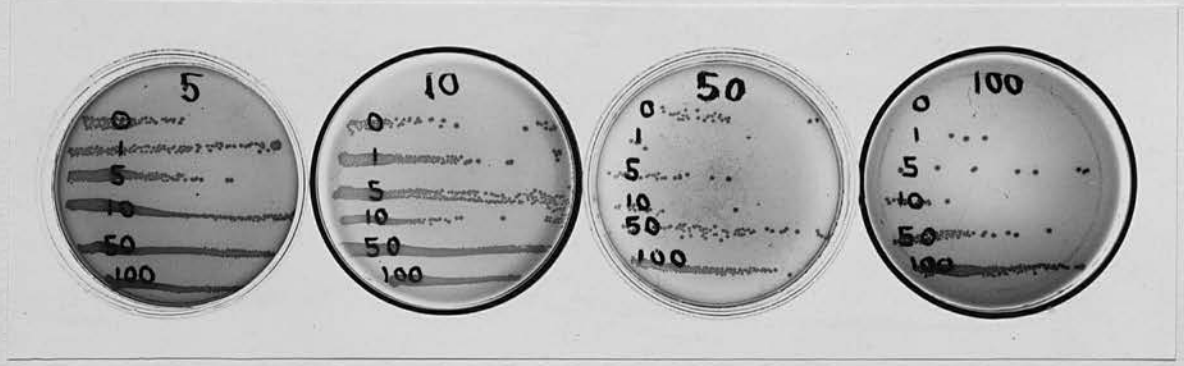


Fig: 27. *A streak plate technique providing evidence of mutation.*

Resistant mutant colonies of various degrees are obtained by putting large inocula on antibiotic-containing plates and picking off the survivors. These colonies are grown overnight in broth to uniform turbidity (Brown No. 4 = 3000 million E. coli per ml.), and loopfuls are streaked in turn on plates containing the antibiotic in the same and higher strengths.

In the photograph cultures from mutant colonies of E. coli 1094 resistant to 0, 1, 5, 10, and 100 μg./ml. streptomycin respectively, are seen to have not only held their resistance but to have mutated still higher as shown by the isolated mutant colonies along these streaks.

Results

The growth after 3 days' incubation at 37°C is seen in the photograph (Fig: 27). It will be seen that each clone has not only maintained its resistance at the appropriate level; but has also mutated again to higher levels. Thus the so-called sensitive clone "0" gives scattered colonies right up to 50 μg/ml., and the reputed "Resistant to 1 μg/ml.," similarly appears sparsely even on 100 μg/ml. The nominal "Resistant to 5", and "to 10 μg/ml." are very comparable to "1", while "Resistant to 50 μg/ml." gives confluent growth on the 2 lower strength plates, and semiconfluent thereafter, while "Resistant to 100 μg/ml." gives confluent growth throughout.

THE REGULARITY OF MUTATIONS IN OTHER GENERA AND SPECIES OF BACTERIA

Although various mutants have been described in strains of Staphylococcus aureus [118], notably the dwarf "G" variants which are still pathogenic [167] and occur naturally at a rate of 1 in 50,000 [180], preliminary experiments with drug-resistant variants of staphylococci suggested that their relative infrequency would make them much less suitable for our studies than Escherichia coli.

Staph. lactis, N.T.C. 8340

Formerly known as Sarcina lutea because of its canary yellow colonies, is a very sensitive organism. It is inhibited by discs of penicillin 1 μg, erythromycin 5 μg., tetracyclines 25 μg., and chloramphenicol 25 μg., is partially sensitive to 10 μg., of streptomycin but resists sulphonamides 200 μg. In fact, as A.T.C.C. 9341, it is used for the commercial assay of Aureomycin, Chloromycetin and penicillin, being sensitive regularly to 0.003 u. of penicillin as compared with the Oxford Staphylococcus needing 0.05 u. Our experiments bore this out:-

Penicillin

TABLE 8

EXPERIMENT 5 *Staph. lactis, N.T.C. 8340, overnight broth culture.*

Conc: of Penicillin u/ml., agar	*Dilu: of inoculum*	*Colony counts at 6 days*					
0 - control	10^6	*64*	*148*	*104*	*100*	*402*	*72*
1	*1*	*0*	*0*	*0*	*0*	*0*	*0*
10	*1*	*0*	*0*	*0*	*0*	*0*	*0*
100	*1*	*0*	*0*	*0*	*0*	*0*	*0*

i.e. Sarcina's penicillin-resistant mutants are extremely rare, none resistant even to 1 u., could be detected in 100 million cocci.

Streptomycin

TABLE 9

EXPERIMENT 6 *Staph. lactis, N.T.C. 8340, overnight broth culture.*

Conc: of Streptomycin μg/ml., agar	*Dilu: of inoculum*	*Colony counts at 6 days*				*Average*
0 - control	10^5	*287*	*270*	*282*	*332*	*293*
1	10^2	*18*	*10*	*17*	*12*	*14*
5	*10*	*12*	*8*	*7*	*14*	*10*
10	*1*	*0*	*0*	*0*	*0*	

i.e. 1 in 46 million resist 1 μg., and 1 in 293 million resist 5 μg.

Streptomycin-resistant Clones

TABLE 10

(a) EXPERIMENT 7 — *Staph. lactis, N.T.C. 8340, "Resistant to 1 μg." subcultured in broth overnight.*

Conc: of Streptomycin μg./ml., agar	*Diln: of inoculum*	*Colony counts at 6 days*	
0 - control	10^6	*249*	*281*
1	10^4	*426*	*443*

i.e. only 1 in 50 breed true "Resistant to 1 μg."

TABLE 11

(b) EXPERIMENT 8 — *Staph. lactis, N.T.C. 8340, "Resistant to 5 μg." subcultured in broth overnight.*

Conc: of Streptomycin μg/ml., agar	*Diln: of inoculum*	*Colony counts at 6 days*				*Average*
0 - control	10^6	*332*	*565*	*360*	*460*	*429*
5	10^6	*1*	*0*	*2*	*1*	*1*

i.e. only 1 in 429 breeds true "Resistant to 5 μg."

One may conclude that Sarcina is sensitive to penicillin because of scanty mutants, and to streptomycin because, though mutants occur they are transient, readily back-mutating to streptomycin-sensitivity.

Staph. aureus, N.T.C. 7447

A typical pathogenic strain with coagulase + golden colonies, was also tested. It was known to be sensitive to antibiotic discs identically to the previous strain, namely sensitive to penicillin, erythromycin, tetracyclines and chloramphenicol, with partial sensitivity to streptomycin and resistance to sulphonamides. The following experiments confirmed this general sensitivity to antibiotics:-

Streptomycin

TABLE 12

EXPERIMENT 9 *Staph. aureus, N.T.C. 7447, overnight broth culture.*

Conc: of Streptomycin μg/ml., agar	*Dilu: of inoculum*	*Colony counts at 6 days*				*Average*
0 - control	10^7	*515*	*334*	*497*	*480*	*387*
		352	*332*	*297*	*289*	
1	10^4	*160*	*222*	*230*	*171*	*196*
5	*10*	*34*	*25*	*14*	*19*	*23*
10	*10*	*2*	*2*	*6*	*10*	*5*
100	*1*	*0*	*0*	*0*	*0*	*0*

i.e. 1 in 2,000 are resistant to 1 μg., 1 in 1½ million are resistant to 5 μg., and 1 in 750 million are resistant to 10 μg.

Streptomycin-resistant clone

TABLE 13

EXPERIMENT 10 *Staph aureus, N.T.C. 7447, "Resistant to 10 μg." subcultured overnight in broth.*

Conc: of Streptomycin μg/ml., agar	*Dilu: of inoculum*	*Colony counts at 6 days*		*Average*
0 - control	10^7	*232*	*222*	*277*
1	10^7	*205*	*223*	*213*
10	10^7	*213*	*225*	*219*

i.e. this "Resistant to 1 μg." clone is breeding true.

Penicillin

TABLE 14

EXPERIMENT 11 *Staph. aureus, N.T.C. 7447, overnight broth culture.*

Conc: of Penicillin u/ml., agar.	*Diln: of inoculum*	*Colony counts at 6 days*				*Average*
0 - control	10^8	*725*	*607*	*629*	*756*	*679*
1	*1*	*0*	*0*	*0*	*0*	
10	*1*	*0*	*0*	*0*	*0*	
100	*1*	*0*	*0*	*0*	*0*	

Streptomycin-resistant clone

TABLE 15

EXPERIMENT 12 *Staph. aureus, N.T.C. 7447, "Resistant to 10 g., streptomycin", subcultured overnight in broth.*

Conc: of Penicillin u/ml., agar.	*Diln: of inoculum*	*Colony counts at 6 days*	
0 - control	10^8	*406*	*345*
1	*1*	*0*	*0*
10	*1*	*0*	*0*
100	*1*	*0*	*0*

i.e. there are no resistant to 1 u. or higher penicillin mutants in at least 67,900 million of the original strain, nor in at least 37,500 million of the "Resistant to 10 µg., streptomycin" clone

There is therefore no evidence of cross-resistance between the two drugs. The comparative scarcity of drug-resistant mutants in both the strains of Staphylococcus tested precluded their use for further mutation rate experiments, and it was therefore decided to confine these to the three strains of Escherichia coli described in the next chapter. Later Mycobacterium tuberculosis will be used for more prolonged laboratory and epidemiological studies. Streptomycin-resistance being the particular mutation or group of mutations which they have in common.

MEASUREMENT OF THE MULTIPLICATION RATE OF BACTERIA

Bacterial reproduction is not just a geometrical progression as the population continually doubles itself by binary fission. In the first place some 10 to 20% of individuals do not survive reproduction; but still more important there is the complexity of the growth curve. The log phase of true multiplication is preceded by a lag phase for cell adjustment, and is followed by a stationary phase, in which congestion limits the numbers and from which autolysis leads to their decline. With pneumococci this occurs in two or three days; but E. coli persist for months.

However, the true generation time, the average life in the actively multiplying period, is constant under optimum conditions. For coliform and anthracoid bacilli 20 minutes is quoted [468], for cocci about half-an-hour, and for Corynebacteria and Clostridia some 40 minutes. Shepard [394] showed that the reproduction rate in tissue cultures for Myco. tuberculosis was no less than 48 hours for the first 1 - 3 days and thereafter 24 hours, and comparison of the visible increase in colony size with that of, say, a diphtheroid suggests that tubercle bacilli do indeed produce a generation a day.

Direct counting of bacteria to estimate their generation rate can be done by incubating a diluted broth culture in a chamber such as is used for blood cells, and counting fields periodically. This is only likely to be accurate if oxygenation is maintained, and also the temperature for a 10°C. fall from 37°C. is said to halve the multiplication of E. coli [468]. It is more practicable to incubate a broth culture at 37°C. with aeration by shaking, which incidentally also disperses the cells. At intervals diluted samples are put in the chamber for counting. This is the method we adopted.

EXPERIMENTS ON MULTIPLICATION TIME

a. EXPERIMENT 13.

Escherichia coli communis, N.T.C. No. 86

Method

Two loopfuls of a culture grown overnight at 37°C. in nutrient broth were inoculated into 3 cc of broth in a bijou screw-capped vial. Incubation at 37°C. continued with the vial gently agitated 100 times per minute in a mechanical shaker.

After $1\frac{3}{4}$ hours a 1 in 8 dilution was made, the diluting fluid consisting of 1% phenol and 0.01% methylene

blue, and counts were made in a Neubauer Chamber using a 1/6" objective. Successive large squares contained:-

74 87 84 99 97 97 114 92 Average 93

Two hours later a 1 in 16 dilution gave counts:-

261 304 318 301 276 296 Average 293

Therefore, multiplication is 3 x 2 (the extra dilution) in 2 hours = 3 per hour = 20 mins. per generation.

(The Neubauer large square contains 16 small squares each of 1/400 sq. mm. x 0.1 mm. deep = $\frac{16 \times 10}{400}$ cmm. = 1/250 cmm. Therefore, in these 2 hours 200,000 organisms per cmm. have become 1,200,000.)

b. EXPERIMENT 14

Escherichia coli communis, N.T.C. No. 1094

Method

The inoculum again consisted of 2 loopfuls of an overnight broth culture incubated in 3 cc. of broth at 37°C. in an agitated bijou vial.

After ½ hour a 1 in 2 dilution was made in diluting fluid and counts made in the Neubauer Chamber.

Three large squares contained:-

28 30 31 Bacilli respectively. Average 30

Incubation and shaking continued, and one hour later a 1 in 16 dilution gave counts:-

28 29 29 Average 29

Therefore, 8 divisions have occurred in 2 hours = 15 minutes each. 24 generations will occupy 6 hours and in 10 hours the progeny could theoretically number a million million.

c. EXPERIMENT 15

Staphylococcus aureus, N.T.C. No. 7447

Method

The test organism was grown overnight at 37°C. in Dubos detergent-containing medium [103, 105, 116] and microscopy showed a general dispersion of the cocci. Four loopfuls were inoculated into a bijou vial containing 3 cc. of equal volumes of nutrient broth and Dubos medium. Incubation continued at 37°C. with the culture gently shaken 100 times per minute.

Two hours later a 1 in 2 dilution was made with diluting fluid and counts made using the 1/6" objective of 3 large Neubauer squares as follows:-

55 58 60 Bacilli Average 58

$2\frac{1}{2}$ hours later a 1 in 16 dilution gave counts of:-

71 70 57 62 64 74 Average 66

Therefore, the multiplication time of this strain is:

$$\frac{5}{2} \times 60 \text{ (time in mins.)} \times \frac{1}{8} \text{ (the diln.)} \times \frac{66}{58} \text{ (the count)} = 21.2 \text{ minutes}$$

CONCLUSIONS

Theoretically the onset of resistance for any organism exposed to an antibiotic could be calculated from the formula:-

Frequency of resistance = Mutation Rate X Multiplication Time

Multiplication in the log phase varies fairly widely between genera, so that a single staphylococcus can yield a visible colony in 8 hours while a tubercle bacillus may take a full day to complete its first division. Even within a species there may be variation so that strains of Mycobacterium tuberculosis range in reproduction rate from 16 to 24 hours in liquid medium, and 18 to 27 hours on solid slopes. The fast-growing H37Rv. produced a visible colony from a single bacillus in 8 days, and, though this speed is partly due to its having less lag phase, the rate of multiplication must be only about 9 hours. Nevertheless these differences in generation time within a species are only some two-or three-fold, whereas the

mutation rates vary, as we have seen, far more greatly. It therefore seems likely that mutagenicity, this tendency to mutate, is the over-riding consideration deciding the likelihood of a micro-organism to produce new epidemic types.

In a chronic disease such as tuberculosis there is the opportunity to study this proneness to mutate in individual strains over many years. The Slope Diffusion Test described in Chapter 10 by providing an even gradient of drug-potency enables mutant tubercle bacilli of varying strength of resistance to be seen as well as the broad picture of the sensitivity of the rest of the bacterial population.

In fact, of course, the development of drug-resistance is not quite such a simple matter of the sequence of straight-forward mutations. Not all the progeny survive, say one in five bacilli dying. Some mutants revert resistant to sensitive by "back-mutation"; while probably the majority not only breed truly resistant but provide mutations in their turn, so that the picture of drug-resistance is cumulatively progressive. To illustrate these points is the purpose of the next Chapter.

CHAPTER 8. PROGRESSIVE MUTATIONS IN THREE TYPED STRAINS OF ESCHERICHIA COLI.

SUMMARY

The principle employed throughout these experiments was to make serial ten-fold dilutions of a 24-hour broth culture of the organism to give measurable counts on plates, appropriately higher dilutions being used for those plates containing nil or low strengths of streptomycin, ranging to undiluted culture for the highest drug concentrations. The plates contained MacConkey's medium with streptomycin solution added at the time of pouring, and eight plates were used of each concentration, and the average count taken.

A typical count, that of E. coli N.T.C. 1094, showed that this strain regularly produced:-

1 in 45,000 individuals resistant to 5 μg./ml., of streptomycin,
1 in 4 million resistant to 10 μg./ml., and
1 in 900 million resistant to 100 μg./ml. of the drug.

If we then repeat the experiment with these resistant mutants in turn as inocule, progressively higher counts are obtained.

a. The "Resistant to 5 μg." clone reproduced its resistance to 5 μg., and yielded 1 in 1½ million resistant to 10 μg., and 1 in 28 million resistant to 100 μg./ml.

b. The "Resistant to 100 μg." clone eventually achieved resistance to no less than 25,000 μg./ml. of streptomycin, and became streptomycin-dependant with only scanty growth on those plates with little or no antibiotic. The fact that such growth was obtained at all, of the order of 1 in 3 million bacilli, was ascribed to 'back-mutation'.

Counts were made of the proportion of resistant bacilli in the strain E. coli var. neapolitana N.T.C. 414 in a similar manner and taken two stages. Streptomycin concentrations of 2 μg./ml., 1 μg./ml., and 0 (control) were used; the broth culture being diluted ten times for the 2 g., plate, 1,000 times for the 1 μg., plate, and 100,000 times for the control plate, these dilutions having been found by previous trial to give manageable counts. It was found that E. coli N.T.C. 414 contained initially 12.2% of bacilli resistant to 1 μg., and 1.8% resistant to 2 μg./ml. When, however, the experiment was repeated with a 24-hour broth culture from a colony chosen at random from the plate containing 1 μg./ml., and spreading a dilution of 1,000 X on fresh 2 μg. plates, and 10,000 X on 1 g. and control plates, it was found that there were now 43.8% resistant to 1 μg., and 8% to 2 μg., a rise in frequency of the resistant variants of approximately fourfold.

The third strain studied was the original type species of Escherich, N.T.C. 86. Here again mutants occurred overnight in fixed numbers diminishing with the drug strength:-

1 in 4 resisting 1 μg.,
1 in 60 " 5 μg.,
1 in 2,000 " 10 μg., and
1 in 25 million " 100 μg.,

and once again they could be 'stepped up' by subculturing clones so that that "Resistant to 5 μg." bred 6% true, and gave 5 times as many mutants resistant to 100 μg., as the parent strain.

Mutant counts with N.T.C. 86, however, are complicated by a second range of colonies appearing two days later at all stages of the experiment. While these too were predictable, there were quite twice as many, if not more. It is suggested that these late-comers were adaptive rather than mutant in origin.

STRAINS

Three National Type Cultures were chosen for their discrete colonies, and conspicuous lactose-fermentation on MacConkey's medium. Simple tests with sensitivity discs showed that they had initial overall sensitivities to streptomycin:-

Strains of Escherichia coli.

E. coli neapolitana

N.T.C. 414

E. coli communis

N.T.C. 1094

E. coli communis

N.T.C. 86

(the original type species of Escherich, from the Lister Institute)

EXPERIMENT 16.

Materials

Escherichia coli var. neapolitana N.T.C. No. 414 was grown in nutrient broth from a freeze-dried culture and tested for any abvious contaminants by spreading on blood agar.

Twelve 500 - ml bottles of MacConkey Agar were prepared according to the formula of Mackie & MacCartney 10th Edn., p.216, and streptomycin hydrochloride added in bulk at 55°C to give two bottles each of 10, 5, 2, 1, ½, and 0 µg/ml of antibiotic. 4" petri plates were poured from the drug-containing medium and stored refrigerated in cans until required.

Method

Preliminary Experiment

The optimum dilution of culture giving countable colonies, below 250 and preferably below 25, was first determined for each strength of streptomycin as follows.

An overnight broth culture of the strain was brought to uniform turbidity by 2 hours' agitation in a mechanical shaker with glass beads, and, after standardising to Brown's opacity No: 4 (3000 million bacilli per ml.),

decimal dilutions were made in sterile saline to 1/million using fresh pipettes at each transfer of 1 ml. into 9 ml. to avoid carrying over undiluted inoculum. 0.1 ml amounts of appropriately diluted culture, as shown in Table 1a, were pipetted onto sets of 4 streptomycin - containing MacConkey Agar plates at each strength of drug. Careful spreading on well-dried plates with an angled glass rod sterilised by flaming is essential not only to avoid colonies becoming uncountable through coalescence; but also to avoid overcrowding producing zones of diminished streptomycin potency, which would allow secondary crops of weaker colonies to confuse the count. The results after overnight incubation at 37°C showed that the optimum dilutions of inoculum for each concentration of streptomycin would be those shown in the second column of Table 16.

Experiment Proper

An overnight broth culture of E. coli neapolitana No. 414 was shaken, standardised, and decimal dilutions made as before. 0.1 ml amounts of culture were taken using the appropriate dilutions indicated by the pilot experiment, and were pipetted, each by its own 1 ml. pipette onto plates of MacConkey Agar with their previously added 10, 5, 2, 1, $\frac{1}{2}$ or 0 μg/ml. of streptomycin. After incubation at 37°C for six days the results shown in Table 16 were obtained.

TABLE 16

FREQUENCY OF RESISTANT INDIVIDUALS IN VARIOUS STRAINS OF ESCHERICHIA COLI

E. coli var. neapolitana, N.T.C. No: 414

Conc: of Streptomycin in µg/ml.	Diln: of Inoculum.	Colony counts at 6 days										Average	Rate per 100 million
0 - Control	10^5	98	96	109	128	125	131	108	125	100	120	112	100 million
½	10^4	16	10	0	0	9	0	7	17	0	20	7.9	705,300
1	10^3	6	25	12	12	11	8	12	27	15	9	13.7	122,300
2	10	10	8	46	10	5	48	17	44	12	8	20.8	1,858
5	3	3	2	7	5	0	4	2	5	0	0	2.8	75.01
10	1	0	0	1	1	10	10	12	10	0	0	4.4	39.29

Results

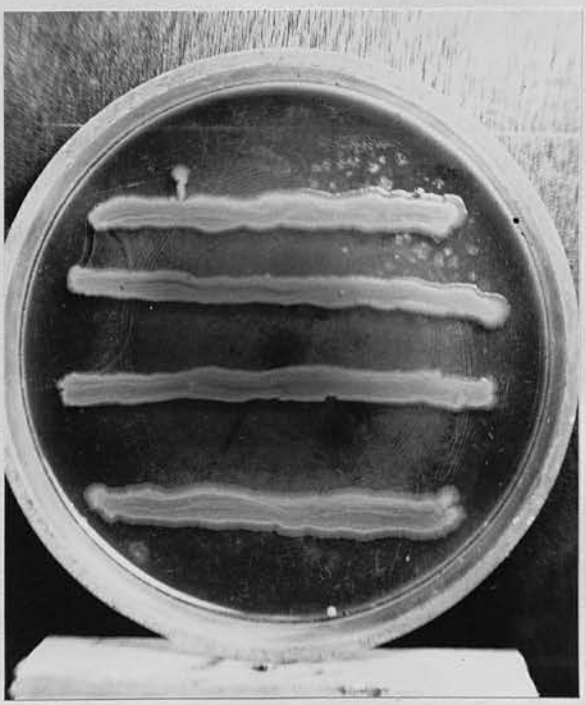
Multiplying the average of the colony counts by the dilution, and dividing by the average number on the control plates, the number of resistant colonies present per 100 million was calculated for each strength of streptomycin in turn. It will be seen that approximately 0.7% of the bacilli can tolerate $\frac{1}{2}$ µg/ml. Streptomycin, about 1 in a thousand can withstand 1 µg., 1.8 in a hundred thousand 2 µg., while 5 and 10 µg. reduce the bacterial population more than a million times - these larger doses being much closer together, however, in the magnitude of their effects.

EXPERIMENT 17.

In order to appreciate fully the progressive development of resistance to streptomycin by this organism it is necessary to see what happens to subsequent generations of these mutants on further exposure to the drug.

In this simple streak-plate experiment colonies of varying degrees of resistance were subcultured in neutral broth from the various plates of the above experiment, and after overnight growth at 37°C were streaked in parallel using a standard loop on MacConkey Agar plates containing higher doses of streptomycin with the results seen in the photograph in Fig: 28.

The progressive breeding of still more resistant mutants among the progeny of each resistant clone is clearly seen. The three resistant clones have not only maintained each their own level of resistance but have produced mutants in turn to higher levels shown by the scattered colonies on the streak lines.



0 μg./ml. streptomycin

Fig: 28. *Progressive mutations in E. coli neapolitana, No.: 414.*

Three resistant and one nominally sensitive colony from earlier experiments are incubated as parallel streaks on streptomycin-containing plates with these results:-

Clone	Streptomycin in plate		
	0	10	100 μg/ml
Sensitive	++++	+	-
Resistant to ¼ μg.	++++	+++	+
" " 2 μg.	++++	+++	++
" " 10 μg.	++++	++++	++

10 µg./ml. streptomycin

Fig: 28. (Cont.)

100 µg./ml. streptomycin

EXPERIMENT 18.

To confirm the fact that the resistant mutants are mutating in turn, and that their numbers are again quantitatively determined, the whole of Experiment 16 was repeated using as inoculum a pure resistant clone.

Materials

A single colony was chosen at random from a plate of E. coli var neapolitana N.T.C. 414 "Resistant to 1 µg/ml. of streptomycin" and was grown in nutrient broth. MacConkey plates containing 10, 5, 2, 1, ½ and 0 µg/ml. streptomycin were prepared in bulk as before.

Method

Decimal dilutions in saline of an overnight broth culture, shaken and standardised, were made to 1/million and 0.1 ml. inocula of various dilutions spread on plates in sets of 4. Using the plates yielding optimal counts of between 25 and 250 colonies as a guide, the experiment was repeated definitively using undiluted overnight culture (Opacity Brown No: 4) for the 10 µg plates, one-tenth dilution for 5 µg, one-thousandth for 2 µg, and one-ten thousandth for the remaining drug strengths. Incubation of the plates was for 6 days at 37°C.

TABLE 17

FREQUENCY OF RESISTANT INDIVIDUALS IN VARIOUS STRAINS OF ESCHERICHIA COLI

E. coli var. neapolitana, N.T.C. No: 414 "Resistant to 1 μg/ml."

Conc. of Streptomycin in μg/ml.	Diln: of inoculum.	Colony counts at 6 days								Average	Rate per 100 million
0	10^4	72	98	78	116	200	120	110	110	113	100 million
½	10^4	36	40	34	44	40	90	70	40	49.25	43.58 million
1	10^4	34	36	75	31	60	45	35	80	49.5	43.8 million
2	10^3	75	74	104	118	112	88	68	88	90.8	8.041 million
5	10	60	30	20	30	27	36	30	76	38.6	34,570
10	1	12	11	10	19	30	20	0	0	12.75	1,128

Results

The results are shown in Table 17. It will be seen that both ½ and 1 μg/ml. can now do little more than halve the population of bacteria, while 8% survive 2 μg, 1 in 3,000 survive 5 μg, and 1 in 100,000 resist 10 μg. It appears that mutations are occurring with increasing frequency, therefore, giving greater degrees of drug-fastness to high concentrations of streptomycin as well as to low. To bring out this feature, Composite Table 18 compares the resistant mutation rates at each level of the original "sensitive" strain with the clone nominally "resistant to 1 μg".

COMPOSITE TABLE 18.

INCREASED FREQUENCY OF RESISTANT INDIVIDUALS IN *E. COLI NEAPOLITANA* N.T.C. 414 EXPOSED TO STREPTOMYCIN

Conc. of streptomycin in μg/ml. MacConkey Plates.	"Sensitive"	"Resistant to 1 μg/ml".
0	100%	100%
½	8%	43%
1	12%	43%
2	1 in 50,000	8%
5	1 in 1½ m.	1 in 3,000
10	1 in 3 m.	1 in 100,000

EXPERIMENT 19.

Materials

Escherichia coli var. communis obtained from National Type Cultures - Strain N.T.C. No. 1094. This was tested for streptomycin-sensitivity using "Sentest" (Evans) tablets, and also for discrete lactose-fermenting colonies on MacConkey's Medium.

MacConkey Agar 4" plates (Mackie and McCartney 10th Edn p.216) containing streptomycin hydrochloride (D.C.(B)L) added to the medium in bulk prior to pouring. For the purpose of the preliminary experiment, 10 500.ml bottles of MacConkey Agar were melted in a Koch's Steamer, and streptomycin solution added while shaking to give the following respective final concentrations:- 2 each of 0, 1, 5, 10, and 100 μg/ml. The $\frac{1}{2}$ litre bottles were distributed in each case into 8 plates, giving 16 of each strength.

For the definitive experiment which followed, a similar range of streptomycin - containing MacConkey medium was prepared; but only in single 500-ml bottles giving 8 plates of each strength.

Method

Preliminary Experiment

This was a pilot experiment to discover the dilutions of inoculum which would be optimal for giving countable colonies.

1 ml was taken by pipette from a 12-hour broth culture of E. coli communis N.T.C. 1094, grown in a bijou vial and shaken for 2 hours with 2 glass beads before sampling to ensure a uniform suspension. Ten-fold dilutions of this were made in a series of 5 x $\frac{5}{8}$" test-tubes by adding 1 ml to 9 ml saline in succession, a fresh oven-sterilised 1 ml pipette being used for each transfer. The same pipettes were used to inoculate the corresponding sets of plates with 0.1 ml each which were then evenly spread by rotating rapidly against a right-angled glass-rod spreader. Table 19 shows the resulting colony counts from empirically chosen dilutions after overnight incubation at 37°C. Those underlined were selected as the most practicable counts for the subsequent experiment.

Experiment Proper

Again the inoculum was an overnight broth culture shaken in a Kahn-agitator for two hours in a bijou vial with two glass beads to ensure a homogeneous suspension. This was standardised to Brown's Opacity Tube No: 2 (1500 million

TABLE 19

PILOT EXPERIMENT TO DISCOVER DILUTIONS YIELDING COUNTABLE COLONIES ON VARIOUS STRENGTHS OF STREPTOMYCIN

Inoculum: *0.1 ml of 12-hour broth culture of E. coli var. communis N.T.C. No. 1094 shaken 2 hours with glass beads. Colony counts are averages of 4 plates.*

Log dilution of inoculum	*Streptomycin concentrations in MacConkey Agar*				
	0	*1*	*5*	*10*	*100 μg/ml.*
0				*<250*	*0*
1			*∞*	*<25*	*0*
2			*∞*	*<5*	*0*
3			*<250*	*0*	*0*
4			*<25*		
5					
6	*<250*	*<250*			
7	*<25*	*<25*			
8	*<5*	*<5*			
9	*0*	*0*			

E. coli per ml.) by adding drops of saline with a Pasteur pipette and re-shaking. Ten-fold dilutions were then made in tubes of saline, and using the pilot experiment as a guide 0.1 ml amounts of appropriate dilutions were pipetted onto the surface of the plates and quickly spread with the angled glass-rod starting from the greatest dilutions and working upwards to minimise carry-over of organisms.

As will be seen from Table 20, the 1 in 10 million dilution sufficed for the 0 (Control) and 1 μg/ml streptomycin plates, 1 in 10 thousand for those with 5 μg/ml, 1 in 10 for 10 μg/ml plates and undiluted broth culture for the plates with 100 μg/ml.

Results

The resulting colony counts are given in the final columns of Table 20 as a proportion of the count on the untreated media. In every case 8 plates were used, counted separately and averaged, allowance being made for the relative dilution of the inoculum. It will be seen that virtually all the population can survive 1 μg/ml of streptomycin; but that 5 μg reduces the number to 1 in 45,000, while only 1 in 4 million can withstand 10 μg, and the rare mutant enduring 100 μg numbers only 1 in 900 million.

TABLE 20

FREQUENCY OF RESISTANT INDIVIDUALS IN VARIOUS STRAINS OF ESCHERICHIA COLI

E. coli var communis, N.T.C. No: 1094

0.1 ml. inoculum of overnight broth culture shaken 2 hours with beads.

Conc. of Streptomycin in μg/ml. MacConkey Agar Plates.	Dilu: of Inoculum in saline.	Colony counts at 6 days								Average	Rate
0	10^7	25	22	19	26	24	24	19	21	22.5	
1	10^7	16	20	22	21	17	25	25	30	22	100%
5	10^4	0	1	1	0	0	0	0	2	0.5	1 in 45,000
10	10	6	6	2	3	9	3	8	7	5.5	1 in 4 m. approx.
100	1	1	0	0	1	0	0	0	0	0.25	1 in 900 m.

EXPERIMENT 20.

Materials:

A single colony was picked off a 5 μg/ml streptomycin-containing plate of the last experiment, and inoculated into broth. This in turn was subcultured in broth the night before the new experiment, and the new culture known as E. coli communis N.T.C. 1094 "Resistant to 5 μg" used as the next inoculum.

Methods

A pilot experiment was again conducted precisely as before to discover the most suitable dilutions of inoculum. These based on the averages of 4 plates and chosen to yield not more than 25 colonies; but as near that number as possible, were found to be 1 in 1 million for all streptomycin strengths upto 5 μg, while 1 in 10 dilution could be expected to suffice for 10 μg, and pure culture would evidently barely suffice to give resistant mutants on 100 μg. See Table 21.

In the subsequent definitive experiment performed as in Experiment 19 using 0.1 ml inocula from ten-fold dilutions in saline of an overnight broth culture, only 5 plates were spread for each strength. Incubation at 37°C was continued for 1 week, the colony counts being found easiest on the sixth day.

TABLE 21

PILOT EXPERIMENT TO DISCOVER OPTIMUM DILUTIONS OF INOCULUM

Inoculum: *0.1 m. of 24-hour broth culture of E. coli communis N.T.C. 1094 "R to 5" shaken 2 hours with glass beads.*

Log dilution of inoculum	*Streptomycin in MacConkey Agar*					
	0	1	5	10	100	μg /ml.
0					0	
1				< 25	0	
2				< 5	0	
3			∞	0	0	
4			∞	0		
5			< 250			
6	< 25	< 25	< 25			
7	< 5	< 5				
8	0	0				
9	0	0				

Results

These were again averaged, adjusted for their dilutions, and the mutation rate of resistant individuals expressed as a percentage, or rate per million respectively, in the total population given by the count on the control plates.

It will be seen from the final column in Table 22 that the whole of this clone of E. coli are now not only surviving 1 μg but also 5 μg of streptomycin, in other words the mutation conferring this degree of resistance is breeding true. In addition there is a progressive rise in the mutation rates giving higher degrees of resistance, the rate for 10 μg/ml of streptomycin being now 1 in approximately 1½ million, and that for 100 μg/ml occurring once in 28 million.

TABLE 22

FREQUENCY OF RESISTANT INDIVIDUALS IN VARIOUS STRAINS OF ESCHERICHIA COLI

E. coli var. communis, N.T.C. No. 1094. "Resistant to 5 μg/ml".

0.1 ml. inoculum of overnight broth culture shaken 2 hours with beads.

Conc. of streptomycin in μg/ml. MacConkey Agar Plates.	Dilu: of inoculum in saline.	Colony counts at 6 days.					Average	Rate
0	10^6	30	32	27	24	28	28.2	
1	10^6	29	26	29	27	32	28.2	100%
5	10^6	22	24	31	25	28	26	100%
10	10	7	20	12	25	25	17.9	1 in 1.5 m. approx.
100	1	0	0	0	0	0	0	1 in 28 m.

EXPERIMENT 21.

Materials:

A single colony of E. coli communis N.T.C. 1094 was now taken from the 100 μg/ml. streptomycin-containing plate of the last experiment, and was grown in nutrient broth for a week. Half-litre bottles of MacConkey Agar were melted as before, five being required for the pilot experiment and five for the experiment proper, streptomycin being added in each case to four of the bottles as 1, 5, 10 and 100 μg/ml shaking meanwhile and then expeditiously pouring the 16 plates needed for the pilot experiment and 5 for the experiment proper at each strength. The fifth bottle of medium provided a parallel set of untreated control plates.

Method

The pilot experiment was conducted exactly as in Experiment 20 with 0.1 ml pipetted inocula from a well-shaken 12-hour broth culture; but it was found, after two abortive attempts with heavily overgrown plates, that dilutions of 1 in 100,000 to 1 in 100 million would be needed in every case to give a scatter of colonies which could be counted. The results read after 3 days' incubation at 37°C are shown in Table 23.

TABLE 23

PILOT EXPERIMENT TO DISCOVER OPTIMUM DILUTIONS OF INOCULUM

Inoculum: *0.1 ml of 12-hour broth culture of E. coli communis N.T.C. 1094 "R to 100" shaken 2 hours with glass beads.*

Counts are averages of 4 plates.

Log dilution of inoculum	*Streptomycin in MacConkey Agar*				
	0	*1*	*5*	*10*	*100 μg/ml.*
0					
1					
2					
3					
4					
5	*<25*	*<25*	*<25*	*<25*	*∞*
6	*<5*	*<5*	*<5*	*<5*	*<250*
7	*0*	*0*	*0*	*0*	*<25*
8	*0*	*0*	*0*	*0*	*<5*

Basing the experiment proper on this the saline dilution chosen for the inoculum was 1 in a million for all the plates. The overnight broth culture of E. coli communis N.T.C. 1094 "Resistant to 100 μg/ml was adjusted to Brown No. 2 opacity, and then diluted tenfold by transferring 1 ml to 9 ml saline six times using 5 x $\frac{5}{8}$" tubes each with its own oven-sterilised 1 ml. pipette. The final pipette was used to drop 0.1 ml inocula onto the 25 MacConkey plates - 5 of each strength - which were then quickly spread, inverted, and incubated at 37°C.

Results

Table 24 shows the resulting colony counts six days later. It will be seen that this streptomycin-resistant clone gives virtually identical counts at all concentrations up to 5 μg/ml, while 10 μg/ml and 100 μg/ml give not fewer colonies as might be expected; but more - approximately half as many again as the 10 μg. and no fewer than 17 times as many on the 100 μg. In other words the colony count is now rising rather than dropping with increasing streptomycin, and we are now dealing with a mutant which is not only drug-resistant but streptomycin-dependant.

TABLE 24

FREQUENCY OF RESISTANT INDIVIDUALS IN VARIOUS STRAINS OF ESCHERICHIA COLI

E. coli var. communis, N.T.C. No. 1094. "Resistant to 100 μg/ml".

0.1 ml. inoculum of overnight broth culture shaken 2 hours with beads.

Conc. of streptomycin in μg/ml. MacConkey Agar Plates.	Diln: of inoculum in saline.	Colony counts at 6 days.					Average	Rate
0	10^6	1	1	2	3	2	1.8	
1	10^6	3	1	1	3	1	1.8	
5	10^6	3	1	2	1	2	1.8	
10	10^6	1	3	3	5	2	2.8	X 1.5 approx.
100	10^6	45	26	34	23	26	30.8	X 17

Conclusion: The mutant is now not only streptomycin-resistant but streptomycin-dependant.

CONFIRMATORY EXPERIMENT 22.

In order to confirm that the mutation was truly streptomycin-dependant, and thus largely irreversible, a further experiment was planned.

Materials

A broth culture of E. coli communis N.T.C. 1094 "R to 100 μg", subcultured from a 100 μg plate of the last experiment, was grown overnight to bring it into the log phase, and used as inoculum.

Twelve plates each of MacConkey Agar with streptomycin incorporated in strengths of 0, 100 and 1000 μg/ml. were prepared.

Methods

Serial tenfold dilutions brought the inoculum down to 1 in 100,000, 1 in a million, and 1 in 10 million respectively, and 0.1 ml amounts of these, pipetted each with its own 1 ml pipette, were dropped onto 4 plates each of the control '0', and the 100 μg. plates. For the 1000 μg. plates 4 received hundred-fold dilution, 4 ten-fold, and 4 undiluted broth culture. Spreading was done in every case working from the highest dilutions back to minimise carry-over of inoculum.

TABLE 25

EXPERIMENT WITH E. COLI COMMUNIS N.T.C. 1094 "R TO 100 μg/ml"

TO CONFIRM STREPTOMYCIN-DEPENDANCE

Log dilution of inoculum	Streptomycin in MacConkey Agar		
	0	100	1000 μg/ml.
0			∞ ∞ ∞ ∞
1			∞ ∞ ∞ ∞
2			∞ ∞ ∞ ∞ (about 20,000)
3			
4			
5	0 0 0 0	∞ ∞ ∞ ∞ (about 600 +)	
6	0 0 0 0	68 81 79 63	
7	0 0 0 0	8 10 11 9	

Conclusion: *Survival count without streptomycin = < 1 in 4,000.*

Results

It will be seen that the clone has now become virtually wholly streptomycin-dependant, there being no survivors on the streptomycin-free plates when plates containing 100 μg/ml of drug and receiving the same inoculum showed uncounted colonies but certainly several hundred. Moreover plates containing 1000 μg/ml of streptomycin also showed vast numbers of colonies, the number being so unexpected that the plates were overinoculated. Comparison of the two sets of streptomycin plates and consideration of the relative dilutions of inoculum, however, suggested that the counts were probably very similar. The conclusion reached was that the strain had achieved resistance to streptomycin even of the order of 1 μmg/ml, combined with overwhelming dependance on the drug.

EXPERIMENT 23.

It was now planned to investigate in more detail both the extent of the streptomycin-resistance and also the completeness or otherwise of the streptomycin-dependance, after 3 weeks' continuous culture of the strain "1094 Resistant to 100 μg/ml Streptomycin" on 3 successive 100 μg/ml plates.

Materials

It seemed likely the broth culture used for the inoculum would itself now need streptomycin to maintain this strain, and this proved to be the case. Overnight broth cultures of E. coli, N.T.C. 1094 "Resistant to 100 μg" were made from a single colony of the last 100 μg/ml plate of the maintenance culture. Three cultures were made with 0, 20 and 80 μg of streptomycin in 5 ml. of broth, each being duplicated. The degree of turbidity showed unmistakeably that growth increased proportionately with increase of drug, and the culture in 80 μg/5 ml showing the greatest growth was adjusted to Brown No: 2 opacity with saline and used as inoculum.

Bottles of MacConkey Agar 0.5 litre were melted and streptomycin added prior to pouring in concentrations of

TABLE 26

PILOT EXPERIMENT TO DISCOVER OPTIMUM DILUTIONS OF INOCULUM, PRIOR TO INVESTIGATING IN THE CLONE E. COLI N.T.C. 1094 "RESISTANT TO 100 µg/ml. STREPTOMYCIN" AFTER THREE WEEKLY SUBCULTURES IN THAT CONCENTRATION:-

1. *Extent of resistance.*
2. *Any reversal to sensitivity and non-dependance.*

Inoculum: *Overnight broth cultures were grown with 0, 20, and 80 µg in 5 ml. broth. 80 µg/5 ml. showed greatest growth, and 0.1 ml. of it was used.*
Counts are averages of 4 plates.

Log dilution of inoculum	*Streptomycin in MacConkey Agar*				
	0	*100*	*1000*	*10,000*	*25,000 µg/ml*
0	*< 5*			*∞*	*∞*
1	*0*				
2	*0*			*< 25*	*< 5*
3	*0*				
4				*< 5*	*0*
5		*< 250*	*< 250*		
6		*< 25*	*< 25*	*0*	*0*
7		*< 5*	*< 5*	*0*	*0*

0; 100; 1000; 10,000; and 25,000 μg/ml. For the preliminary experiment 20 plates of each strength were poured.

Method

Tenfold dilutions of inoculum up to 1 in 10 million were made in 5 x $\frac{5}{8}$" test tubes using a fresh oven-sterilised 1 ml pipette for each successive transfer of 1 ml into 9 ml saline. The pipettes were left in the tubes and used in reverse order to place 0.1 ml of their appropriate dilutions, well shaken, onto sets of 4 plates as shown in the table 26. After immediate spreading the plates were incubated at 37°C for 24 hours with the results shown.

Using this pilot experiment as a guide the dilutions of inoculum chosen for the definitive experiment were 1% for the 10,000 and 25,000 μg/ml plates, 1 in a million for the 100 and 1,000 μg/ml plates and undiluted inoculum for the plain untreated plates. An overnight culture in 5 ml broth containing 80 μg/ml streptomycin was again prepared, shaken 2 hours with two glass beads in a bijou vial, diluted to Brown No: 2 opacity, and then passed through serial tenfold dilutions to obtain those required. 250 ml amounts of MacConkey Agar received the streptomycin in bulk for each concentration, and 4 plates poured for each strength 0; 100; 1,000; 10,000; and 25,000 μg/ml. The 0.1 ml inoculum was quickly spread, incubated at 37°C for 6 days, and colonies

TABLE 27

FREQUENCE OF RESISTANT INDIVIDUALS IN VARIOUS STRAINS OF ESCHERICHIA COLI

To show 1. Extent of resistance.

2. Any reversal to sensitivity and non-dependence

E. coli var. communis, N.T.C. No. 1094 "Resistant to 100 μg/ml. Streptomycin". 0.1 ml. inoculum of overnight Streptomycin broth culture shaken 2 hours with beads. *Cultures 3 weeks on 3 successive 100 μg plates*

Conc. of streptomycin in μg/ml. MacConkey Agar Plates	*Dilu: of inoculum in saline*	*Colony counts at 6 days*	*Average*	*Rate*
0	*1*	*3 5 6 4*	*4.5*	*1 in 3 million*
100	*10^6*	*11 15 18 8*	*13*	*100%*
1,000	*10^6*	*11 12 9 13*	*11*	*84%*
10,000	*10^2*	*15 22 20 10*	*16.7*	*1 in 10,000 approx.*
25,000	*10^2*	*3 0 1 2*	*1.5*	*1 in 100,000 approx.*

Conclusions: *Only 1 in 3 million have reverted R. & D. → S.*

Nearly all are R. to 100 and also to 1,000 μg.

1 in 10,000 are R. to 10,000 μg, and 1 in 100,000 are R. to 25,000 μg.

" R = Resistant, D = Dependant, S = Sensitive"

resulting counted and recorded in Table 27. After averaging and making allowance for the dilution factor in each case the mutation rates shown in the last column were obtained.

It will be seen that if we use the 100 μg/ml streptomycin now as the control plate, no less than 83% of these bacilli are also resistant to 1,000 μg/ml, and mitants are occurring resistant to 10,000 and 25,000 μg/ml at rates of 1 in 10,000 and 1 in 100,000 approximately, which are far higher than the back-mutation rate to streptomycin-sensitivity, which is only of the order of 1 in 3 million.

The Composite Table 28 summarises Experiments 1 to 5, and shows clearly the progressively higher mutation rates occurring in the already resistant clones of E. coli surviving streptomycin. Back-mutation, while it does occur, is obviously relatively much less frequent, and with the natural selection which will happen so long as the drug is administered, it is easy to see how antibiotic resistance occurs in a rapidly steepening curve so characteristic of streptomycin, and how only prolonged cessation of treatment will give reversal to sensitivity again.

COMPOSITE TABLE 28

FREQUENCY OF RESISTANT INDIVIDUALS IN VARIOUS CLONES OF E. COLI COMMUNIS N.T.C. 1094

Concn. of streptomycin in μg/ml. MacConkey Plates.	'Sensitive'	"Resistant to 5 μg/ml"	"Resistant to 100 μg/ml	"Resistant to 100 μg/ml., 3 x subcultural"
0	All	All	6%	1 in 3 m.
1	All	All	6%	-
5	1 in 45,000	All	6%	-
10	1 in 4 m.	1 in 1½ m.	9%	-
100	1 in 900 m.	1 in 28 m.	All	All
1000	-	-	-	84%
10,000	-	-	-	1 in 10,000
25,000	-	-	-	1 in 100,000

STREAK-PLATE DEMONSTRATIONS OF STREPTOMYCIN-DEPENDANCE

To demonstrate graphically the reality of the streptomycin-dependance achieved by this strain of E. coli some simpler experiments were done using streak-plates and these were photographed by transmitted light to show up any trace of growth as a silhouette.

EXPERIMENT 24.

In the first experiment, Fig: 29, five clones of streptomycin-sensitive or resistant E. coli communis N.T.C. 1094, each derived from a single colony growing on a MacConkey plate containing the appropriate concentration of the drug, were suspended in 5 ml of broth and a loopful of the suspension used as inoculum in each case. The inocula were streaked in parallel on MacConkey plates containing 0, 1, 5, 10, and 100 μg/ml streptomycin, two rows of such plates being used to duplicate the whole experiment.

It will be seen that the three least resistant clones, those "Resistant to:- 0, 1, and 5 μg/ml respectively" grow on all the three lower strength plates, those containing 0, 1, and 5 μg. but there was no growth beyond this point. (N.B. The plates unfortunately read from right to left. The labelling on each plate is correct however, having been made to read by transmitted light

through the plates). The "Resistant to 10 μg/ml" clone, as might be expected grows on a plate higher to reach its own level of 10 μg. But when we come to the "Resistant to 100 μg/ml" clone of E. coli. 1094 we find the very different picture of no growth at all on any lower strength plate than 100 μg/ml streptomycin, showing the necessity of the antibiotic for its continued growth.

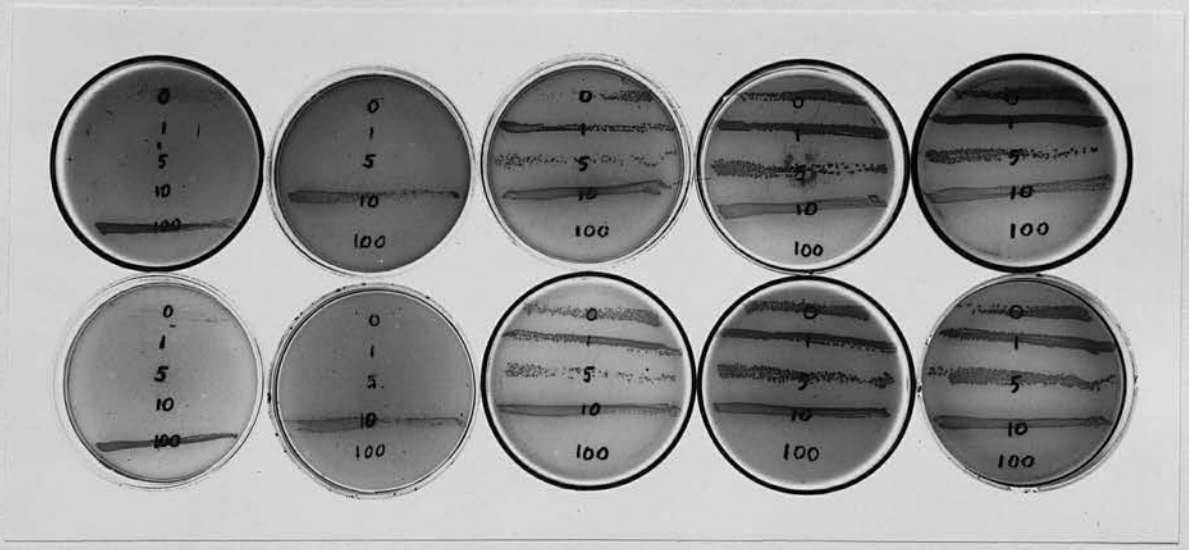


Fig: 29. *Demonstration of the Streptomycin-dependance of the strain "E. coli, No: 1094, Resistant to 100 µg. streptomycin".*

Resistant clones from previous experiments were incubated as parallel streaks on the following streptomycin-containing plates:-

Nominal resistance of clone	*Concentration of streptomycin in plate*				
	100	*10*	*5*	*1*	*0 µg/ml*
0 µg.	-	-	+	+	+
1 µg.	-	-	+	+	+
5 µg.	-	-	+	+	+
10 µg.	-	+	+	+	+
100 µg.	+	-	-	-	-

It will be seen that the three "weaker" clones grow only up to 5 µg./ml., that the "Resistant to 10 µg.", grows on a plate higher i.e. up to its nominal level of 10 µg./ml.; but that the "Resistant to 100 µg." clone is only able to grow on the 100 µg./ml streptomycin plate.

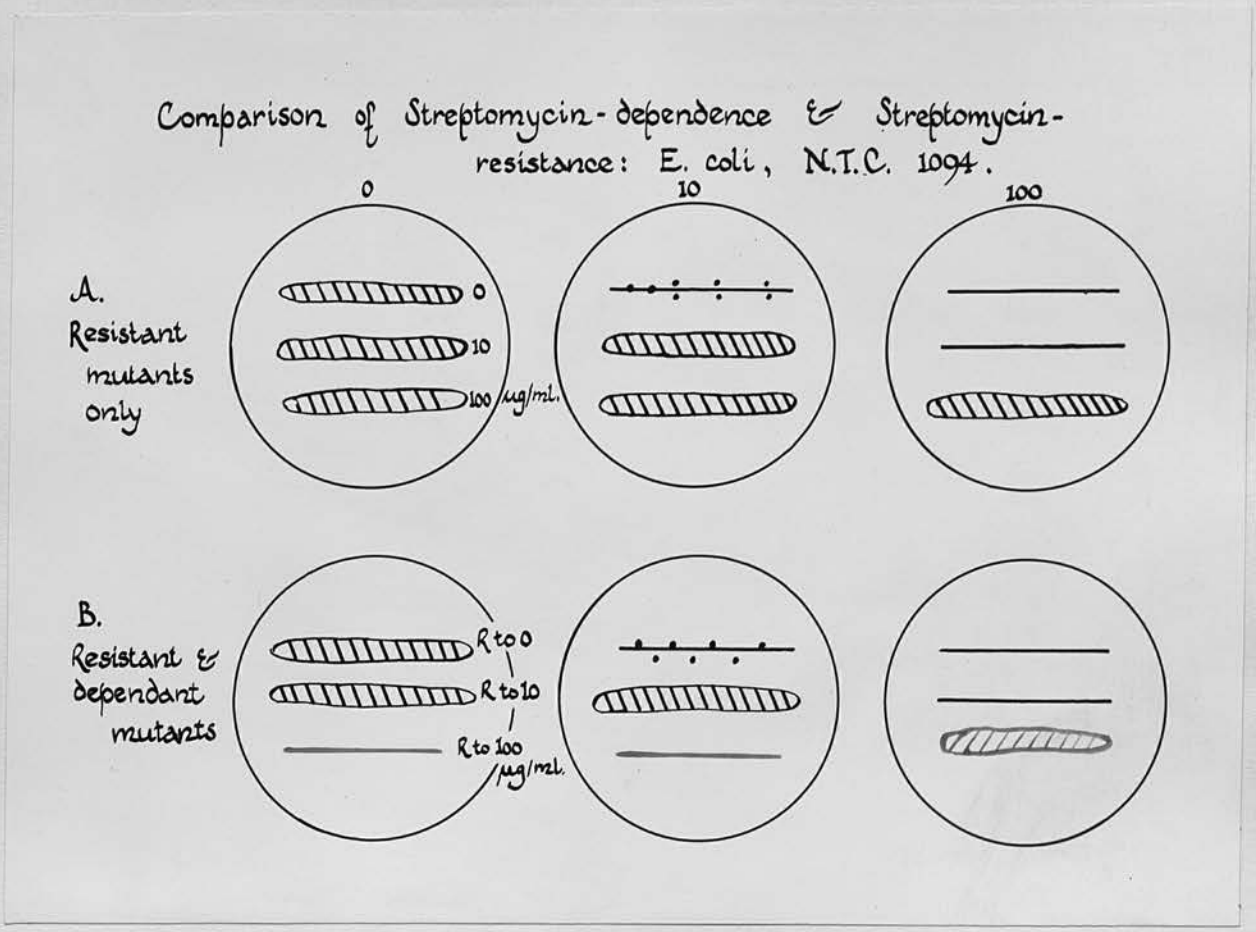


Fig: 30. *This simplified diagram, based on the phenomenon seen in the foregoing Fig: 29, shows the complicated picture which results when graduated resistance to streptomycin is superimposed on dependance operating in the reverse direction (shown in red). The superimposition of multiple phenomena of this sort can reach great complexity as shown in Fig: 32.*

EXPERIMENT 25.

Fig: 31 is a still more conspicuous demonstration of this streptomycin-dependance phenomenon. The clone E. coli. 1094 "Resistant to 100 μg." was suspended in broth and diluted 1 in 1 million with saline from the suspension adjusted to Brown 2 opacity. 0.1 ml amounts were inoculated onto three MacConkey plates containing 10, 100 and 1,000 μg/ml streptomycin, each plate being repeated in triplicate. It will be seen that the colony counts increase progressively as the drug concentration rises, the fact that there is any growth at all at the lower levels suggesting that some back mutation is occurring; but being probably exaggerated by the crudity of the method.

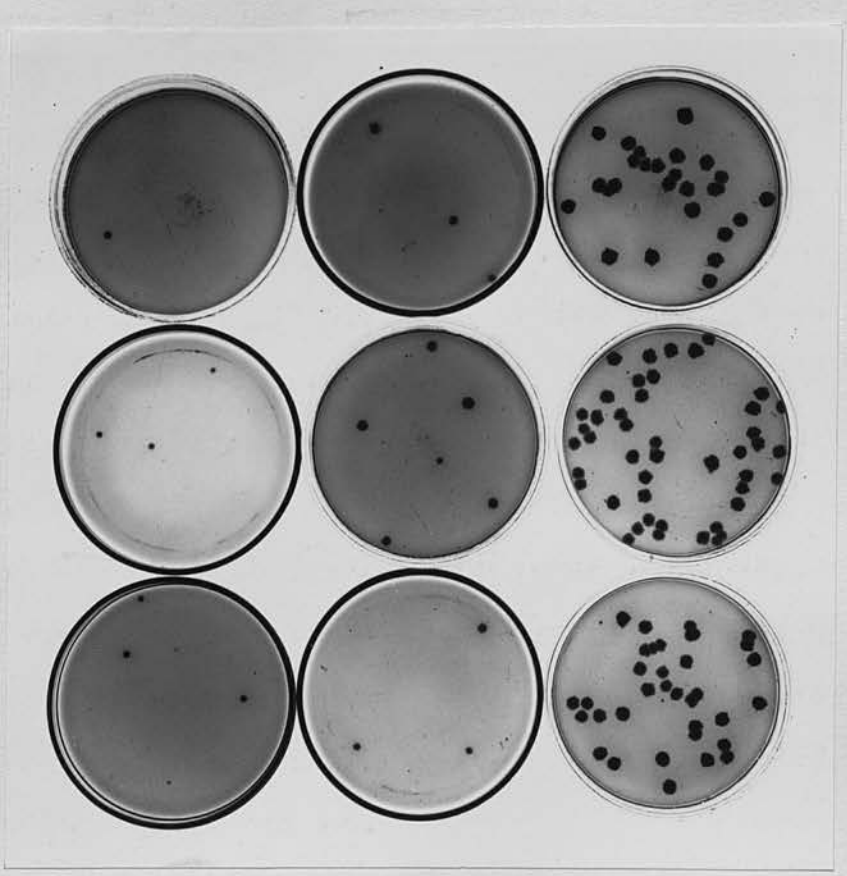


Fig: 31. *Another demonstration of streptomycin-dependance.*

The clone "E. coli No. 1094, Resistant to 100 μg. Streptomycin", obtained from previous experiments, is now giving progressively higher counts with increasing amounts of streptomycin.

Reading from left to right:-

A uniform inoculum yields 1 - 3 colonies on agar with 10 μg./ml. streptomycin.

" 3 - 6 colonies on agar with 100 μg./ml. streptomycin.

" 27 - 44 colonies on agar with 1000 μg./ml. streptomycin.

EXPERIMENT 26.

It is interesting to speculate whether the administration of streptomycin could actually lead to a relapse of infection in a subject harbouring a strain of E. coli. which had become drug-dependant as in the in vitro tests described above. The following in vivo experiment was an attempt to detect this.

Nine white mice of average weight 10 G. were marked distinctively with dyes and distributed into one group of 5, and a control group of 4. The group of 5 each received 1 mg. Streptomycin hydrochloride dissolved in 0.5 ml distilled water by intraperitoneal injection on each of three successive days. One of these mice received nothing more. The remaining 4, and the control group of 4, were also injected intraperitoneally with suspensions of an overnight streptomycin-broth culture of E. coli. communis No.1094 "Resistant to 25,000 μg/ml" harvested from Experiment 23. This was adjusted turbidimetrically to Brown Opacity Tube No: 4 (3,000 million organisms/ml) and then serially diluted so that pairs of mice, one untreated, the other pre-treated with streptomycin, received respectively 150 million E. coli., 15 mil., 1.5 mil., and 0.15 mil. As no disease was yet apparent in any of the mice, all the 8 infected animals (both treated and untreated with streptomycin) were given

a massive dose of 1,500 million E. coli. i.p. Three out of four of both groups, those having streptomycin and those not, succumbed in 24 hours. The survivor in each group was the mouse which had previously had the biggest innocula of 150 mil. organisms. The experiment was therefore inconclusive, largely no doubt because attenuation during the successive in vitro experiments had made all but overwhelming infection abortive, and even that could be withstood by the two survivors whose previous injections had presumably been adequate to pre-immunise them. But while no streptomycin-dependance had been demonstrated, the streptomycin-resistance of this strain of E. coli., which we have seen in vitro, had had suggestive confirmation in vivo also, for 3 daily doses of 1 mg. (i.e. 1/10,000 of the entire body weight) had not sufficed to prevent a potentially fatal infection.

EXPERIMENT 27.

Under natural conditions, of course, it must come about that quite a number of different mutations are occurring in any one strain of bacteria simultaneously, and this muct be particularly so with a drug such as streptomycin to which resistant mutations so readily occur. The following experiment gives an indication of how complex the cumulative effect can become.

Materials

Colonies of E. coli. communis N.T.C. 1094 picked off drug-containing plates of the previous experiments 1 - 5 were grown separately in broth overnight, 80 μg/ml of streptomycin being added to the clones from 1,000; 10,000; and 25,000 μg plates to satisfy their drug-dependance. The resulting cultures provided inocula nominally "sensitive" and "resistant to 1; 10; 100; 1,000; 10,000 and 25,000 μg/ml drug respectively". Plates of MacConkey Agar were prepared containing all these strengths of streptomycin.

Method

The seven inocula were all streaked in parallel on all seven plates, incubated overnight at 37°C and photographed in silhouette (Fig: 32) to show up any trace of growth. The 1 μg/ml plate has been excluded as it proved identical to the control (0 μg/ml streptomycin).

Fig: 32. *Resistant mutants of various degrees of E. coli 1094 streaked on drug plates to show diverse properties:-*

Clone:- *Plate:-*	*0*	*5*	*10*	*50*	*100*	*25,000*	*μg/ml. streptomycin*
'Resistant to 0'	*+++*	*+++*	*0*	*0*	*0*	*0*	
' " to 1'	*+++*	*+++*	*0*	*0*	*0*	*0*	
' " to 10'	*+++*	*+++*	*+++*	*0*	*0*	*0*	
' " to 100'	*0*	*0*	*0*	*+++*	*+++*	*+++*	
' " to 1000'	*+++*	*+++*	*+++*	*++*	*+++*	*+++*	
' " to 10,000'	*0*	*0*	*0*	*+++*	*+++*	*+++*	
' " to 25,000'	*+*	*++*	*++*	*++*	*+++*	*+++*	

This suggests:-

"R to 0 & 1" are alike R to 5 and no further.

"R to 10" is R to 10 and no further.

"R to 100 & 10,000" are Strep-dependant & R between 50 - 25,000.

"R to 1000 & 25,000" are similarly R but not completely dependant.

N.B. *R = Resistant* (μg)

Results

A study of the photograph (Fig: 32) will show that the three less resistant mutants are unable to survive more than small amounts of streptomycin, the limit being 5 μg for the 'sensitive' and 'resistant to 1 μg' and 10 μg for that 'resistant to 10 μg'. The more resistant mutants on the other hand grow as well or better on the more strongly dosed plates, the 'resistant to 100 μg' and 'resistant to 10,000 μg' clones being fully dependant so that they do not survive below 50 μg concentration of streptomycin, while the 'resistant to 1000 μg' strain shows a suggestion of a biphasic curve with least growth at the intermediate point between sensitivity and dependance, while the most resistant clone of all, that to 25,000 μg, has been back-mutating sufficiently to give some growth on all plates but diminishing inversely to the drug-concentration. The bewildering overall picture which could emerge from all these, and no doubt many other, diverse mutants all superimposed is obvious. All that we can say clinically is that our drug is introducing bias into the normal to and fro mutations so that, temporarily at least, more pro-resistant mutations than others will be encouraged, and to progressively higher levels, by a process of natural selection.

COMBINED ADAPTATION AND MUTATION

EXPERIMENT 28.

The previous nine experiments with the type culture E. coli. communis No: 1094 show clearly that drug resistance to the highest degree can result from the selective breeding of a few resistant organisms present in the original stock. Even those variants which are excessively rare could, with the enormous reproduction rate of bacteria, periodically get the chance to replace the normal population.

But the wide-spread emergence of drug-fast strains, including some apparently initially resistant, and also those resistant to more than one antibiotic and even several simultaneously, suggests that with some bacteria another and additional factor contributes, namely that some individual bacteria are capable of adaptation of their enzyme systems to nullify the effects of the antibiotic. The time needed for such adaptations, however short, can never be quite so instantaneous as a mutation and it seemed possible that in this way a distinction could be made between the two phenomena.

Materials

E. coli. communis N.T.C. No. 86 was selected as it had been noticed with this organism that plate counts of

Fig: 33. *Plates showing that drug-resistance in the strain E. coli, N.T.C. No: 86 , is probably due to both adaptation and mutation.*

Left: *A control plate of agar with no streptomycin. It has grown 17 colonies all alike.*

Right: *An agar plate with 2 μg./ml. streptomycin. This has one large colony, which appeared at 6 hours and is probably a mutant.*

But there are also 17 small colonies which appeared only at 3 days, suggesting adaptation to the drug.

streptomycin-resistant colonies had been increasing inexplicably over the passage of several days. MacConkey Agar $3\frac{1}{2}$" plates were used incorporating 2 μg/ml streptomycin hydrochloride added at 55°C just before pouring. Plain MacConkey Agar plates provided controls.

Method

An overnight broth culture incubated at 37°C of E. coli. 86 was shaken for 2 hours with 2 glass beads in a bijou vial and diluted 1 in 10 million with saline. 0.1 ml of this was spread on the 2 μg/ml streptomycin and the plain plates with a glass rod and the plates incubated at 37°C for 1 week with careful daily examination.

Results

Fig: 33 shows photographs after 3 days of a pair of equally inoculated plates, that on the left containing no streptomycin, that on the right 2 μg/ml. Both plates show comparable numbers of colonies (17 and 18 respectively); but on the streptomycin plate the colonies are of two sorts - a single large colony which had been visible to the naked eye since the first day, and was therefore presumably a true mutant, while the other 17 colonies on this plate were late developers only appearing after three days, and therefore considered to be adapters.

EXPERIMENT 29.

It is possible of course, that the ability to modify an enzyme system and thus avoid inhibition by an antibiotic, is itself conferred by a mutation at least when demanded in high degree by greater concentrations of streptomycin than that with which we were dealing in Experiment 10. If this be so drug-resistant 'adaptors', and 'mutators' pure and simple, should both be occurring at fixed rates. The following experiment shows that this is so.

Materials

E. coli. communis N.T.C. No. 86 grown overnight in nutrient broth to bring it into the logarithmic phase of growth was shaken for 2 hours with glass beads and adjusted to Brown No: 4 opacity tube (3,000 mil organisms per ml). MacConkey Agar was prepared in ½ litre bulks and streptomycin hydrochloride added at the time of pouring to give sets of plates with 0, 1, 5, 10 and 100 μg/ml of antibiotic.

Pilot Experiment

Method

To discover suitable dilutions of inoculum to give practicable colony counts the broth culture of E. coli. No:

TABLE 29

PILOT EXPERIMENT TO DISCOVER OPTIMUM DILUTIONS OF INOCULUM

Inoculum: *0.1 ml overnight broth culture of E. coli communis N.T.C. No. 86 shaken 2 hours with glass beads,*

Counts are average of 4 plates.

Log dilution of inoculum	*Streptomycin in MacConkey Agar*					
	0	*1*	*5*	*10*	*100*	*μg/ml.*
0					*< 25*	
1					*< 5*	
2					*0*	
3				*<250*	*0*	
4			*<250*	*< 25*		
5			*< 25*	*< 5*		
6	*<250*	*<250*	*< 5*	*0*		
7	*< 25*	*< 25*	*0*			
8	*< 5*	*< 5*				
9	*0*	*0*				

Plates were read after 4 days, and it was noticed that about half the colonies took 2 days to become visible to the naked eye.

86 was taken through serial tenfold dilutions to 10^9 using fresh oven-sterilised 1 ml pipettes for each transfer into 9 ml sterile saline. The same pipettes imoculated the plates with 0.1 ml inocula in the manner shown in Table 8a, and the entire set of plates was in quadruplicate. The plates were spread rapidly by rotating an angled glass-rod, and were then incubated at 37°C.

Results

Table 29 shows the approximate colony counts, averaged from each 4 plates, after 4 days. It was noticed even at this stage that about half of all these colonies had not appeared during the first two days' incubation. Counts of about twenty colonies gave the inoculum dilutions chosen for the definitive experiment.

Experiment Proper

Method

Using the same materials as the previous pilot experiment, and it as a guide, the broth culture of E. coli 86 "Sensitive to streptomycin" was diluted 10^7 for the control and 1 μg plates, 10^5 for the 5 μg plates, 10^4 for 10 μg and used undiluted for those containing 100 μg. Four plates of each strength of streptomycin - containing MacConkey Agar were used, and after incubation at 37°C colony counts

TABLE 30

FREQUENCY OF RESISTANT INDIVIDUALS IN VARIOUS STRAINS OF ESCHERICHIA COLI

E. coli var. communis, N.T.C. No. 86 "Sensitive to streptomycin".
0.1 ml. inoculum of overnight broth culture shaken 2 hours with beads.

Conc. of streptomycin in μg/ml MacConkey Plates	Diln. of inoculum in saline	Colony counts at 1 day (upper nos.) and 3 days (lower nos.)				Average	Rate
0	10^7	19	17	13	13	15.5	
		19	17	13	13	15.5	
1	10^7	1	6	4	3	3.5	25%
		17	19	11	16	15.5	100%
5	10^5	31	22	25	29	27	1 in 60
		53	45	49	50	49	1 in 30
10	10^4	7	8	6	9	7.5	1 in 2000
		77	57	63	68	66	1 in 250
100	1	5	8	6	7	6.5	1 in 25 m.
		13	21	10	17	15	1 in 10 m.

Upper Numbers = Mutation Rate
Lower Numbers = Adaptation Rate ?

were made at 24 hours and again at 3 days. These were averaged, adjusted for the dilution factor, and two separate rates obtained.

Results

These are shown in Table 30, the black being the immediate number of resistant colonies, and therefore straightforward resistant mutants, while the red totals representing a quite different but nonetheless equally regular rate suggest a slower adaptation, possibly itself inaugurated by a mutation. It will be seen that this second mechanism conferring streptomycin resistance on the strain E. coli 86 is occurring more frequently throughout than the straight mutation, being 4 times commoner to 1 μg, twice as common at 5 and 100 μg, and 8 times more so at 10 μg streptomycin.

TABLE 31

PILOT EXPERIMENT TO DISCOVER OPTIMUM DILUTIONS OF INOCULUM

Inoculum: *0.1 ml overnight broth culture of E. coli communis N.T.C. No. 86 "Resistant to 5 μg/ml. streptomycin", shaken 2 hours with glass beads.*

Counts are average of 4 plates.

Log dilution of inoculum	*Streptomycin in MacConkey Agar*					
	0	*1*	*5*	*10*	*50*	*100*
0					*< 250*	*< 250*
1					*< 25*	*< 25*
2					*< 5*	*< 5*
3					*0*	*0*
4						
5			*< 250*	*< 250*		
6	*< 250*	*< 250*	*< 25*	*< 25*		
7	*< 25*	*< 25*	*< 5*	*< 5*		
8	*< 5*	*< 5*	*0*	*0*		
9	*0*	*0*				

EXPERIMENT 30.

This was a limited experiment with a "resistant to 5 μg" clone from a true mutant colony on the 5 μg plates of Experiment 11 to see whether both simple mutation and adaptation continue at fixed but still greater rates on further subculturing.

Materials

An overnight broth culture of E. coli communis N.T.C. 86 "Resistant to 5 μg/ml streptomycin" was the inoculum, and MacConkey Agar plates were poured with streptomycin added in only 5 μg/ml and 100 μg/ml concentrations.

Methods

A preliminary pilot experiment showed that optimum dilutions of the inoculum in saline would be 1 in 10 million for the control plates, 1 in a million for the 5 μg plates and 1 in 10 for 100 μg. (See Table 31).

In the actual experiment 4 plates were used at each of these strengths and were spread with an overnight broth culture dispersed with beads and serially diluted with saline to these amounts. After incubation at 37°C colony counts were made at 1 day and 3 days and separately recorded.

Results

Table 32 shows that separate "mutation" and 'adaptation' rates continue to operate regularly, and are progressively rising in frequency at both the 5 and 100 μg. levels, suggesting that both mechanisms depend on natural laws and may well both in fact originate from mutations. Thus the two rates at the 5 μg. level have risen from scarcely 2% and 3% to 6% and 90% respectively, while those at the 100 μg level, formerly 1 in 25 mil. and 1 in 10 mil., are now 1 in 5 mil. and 1 in ¾ million respectively.

EXPERIMENT 31.

In the previous experiment we were dealing with the rates for further "adaptors" and "mutators" within a single drug-resistant clone - that to 5 μg. streptomycin. In this experiment an attempt was made to compare side by side on streak plates the behaviour on subsequent sub-cultures of the two clones themselves - "adaptor clone" and "mutator clone", and that was done for pairs of colonies from the three levels of streptomycin resistance 5 μg, 10 μg, 50 μg.

Materials

E. coli No: 86 drug-resistant colonies were selected from previous experiments, first-day large colonies providing the outright "mutants", and small late growers the so-called "adaptors". Overnight broth cultures were grown of these pairs for the three degrees of resistance - 5, 10 and 50 μg. streptomycin, and also the original sensitive strain.

Method

Standard loopfuls of the sensitive strain and the three degrees of resistant "mutants" were streaked side by side with the corresponding three "adaptors" 'A' on 4 MacConkey Agar plates containing 0, 10, 50 and 100 μg/ml streptomycin respectively.

TABLE 32

FREQUENCY OF RESISTANT INDIVIDUALS IN VARIOUS STRAINS OF ESCHERICHIA COLI

E. coli var. communis, N.T.C. No. 86 "Resistant to 5 μg/ml. streptomycin".

0.1 ml. inoculum of overnight broth culture shaken 2 hours with beads.

Conc. of streptomycin in μg/ml. MacConkey Agar	Dilu. of inoculum in saline	Colony counts at 1 day (upper nos.) and at 3 days (lower nos.)				Average	Rate
0	10^7	14	8	9	11	10.5	
		14	8	9	11	10.5	
5	10^6	7	8	7	5	6.75	6/7
		75	101	111	89	94	90%
100	10	2	6	0	0	2	1 in 5 ml
		23	28	16	23	17.5	1 in ¾ ml.

Results

The growth after 24 hours at 37°C showed that the resistant progeny at 24 hours are virtually identical for both types of resistant colony apart from some minor inconsistency at 10 μg. where the "true mutator" gave a confluent growth and the "adaptor" seemed to show some partial reversal of drug-resistance. The fact that both "mutator" and apparent "adaptor" continued to breed resistant strains suggests that both in fact originate in genotypic mutations.

CHAPTER 9. ANOMALOUS FINDINGS AND FALLACIES

1. OVERLOADING OF ANTIBIOTIC

Maass and Johnson [274, 275] estimated that each stephylococcus binds some 750 molecules of penicillin thereby weakening the residual antibiotic in a solution. It seemed likely that a similar quantitative loss occurs with streptomycin in contact with coliform bacilli, and the following experiment shows that this is so, and that very high colony counts (500-1000 colonies) are false evidence of drug-resistance.

EXPERIMENT 32.

The clone of E. coli communis No. 1094 "Resistant to 25,000 μg" was taken from previous experiments and maintained in nutrient broth containing streptomycin 80 μg/ml. An overnight subculture in this medium was adjusted to Brown No. 4 opacity (3,000 mil. organisms/ml). After 2 hours' agitation in a vial with glass beads, half the inoculum was diluted 1 in 100 the remainder being kept undiluted.

Identical MacConkie Agar plates containing 25,000 μg/ml streptomycin hydrochloride were poured, and 0.1 ml inocula of the two different strengths of culture pipetted onto each, using

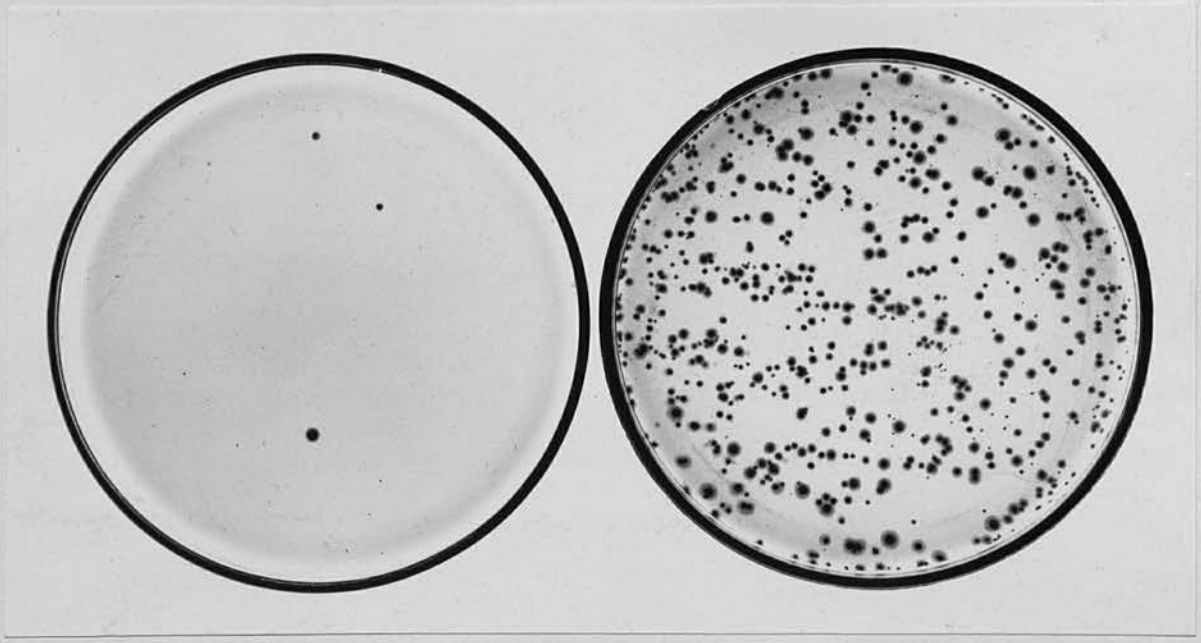


Fig: 34. *The spuriously high count of "resistant" colonies caused by overloading an agar plate containing 25,000 μg./ml. of streptomycin with an excessive inoculum of the clone E. coli No: 1094 "Resistant to 100 μg."*

Left: *Using optimum inoculum (0.1 ml. of 1% overnight broth culture) the yield is 3 truly mutant colonies.*

Right: *Using one hundred times this amount the yield is many more than 300, and the colonies show no uniformity in size, suggesting that the appearance of many is due to mere exhaustion of the streptomycin in the plate.*

separate sterile pipettes, the spreading also being done by separate sterile angled glass-rod spreaders. After overnight growth at 37°C the resulting colonies are shown in silhouette in Fig: 34. It will be seen that the colony counts were 3 for the 1 in 100 dilution; but approximately 750 for the undiluted culture. Moreover the colonies on this overloaded plate were of many sizes and even in clusters.

EXPERIMENT 33.

In order to confirm that this latter high count is in fact spurious, subcultures were tested as follows. Four MacConkie Agar plates containing 0; 1000; 10,000; and 25,000 μg/ml of streptomycin respectively were prepared, and overnight cultures made to give inocula from colonies from the two plates of the previous experiment. These were grown in broth to which streptomycin 80 μg/ml had been added, and after 12 hours the two cultures were carefully adjusted to an equal turbidity, Brown Opacity Tube No. 4.

'A' is the clone from one of the 3 mutants in the left-hand culture (Fig: 35) and 'B' is from one of the 750 suspect colonies of the right-hand culture. 'A' and 'B' are streaked in parallel on the four media and grown overnight at 37°C. The results (Fig: 35) show that while both are streptomycin - dependant as they do not grow on the plain

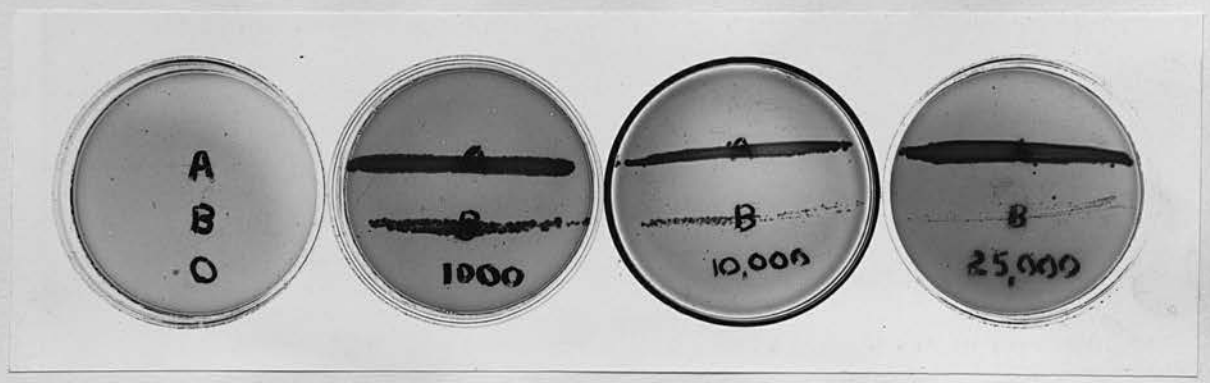


Fig: 35 *Streak sub-cultures from the plates of the previous experiment. 'A' is from the colonies of the left-hand plate (Fig: 34), 'B' from the right-hand one. The two inocula have been incubated on agar plates containing 0; 1,000; 10,000; and 25,000 μg./ml. streptomycin.*

'A' is seen to be truly streptomycin-dependant and highly resistant (to 25,000 μg./ml.)

'B', however, is streptomycin-dependant as it will grow on the control plate; but is only moderately resistant (± to 10,000 μg.).

control plate, and both are to some degree streptomycin-resistant, only the 'A' strain is truly resistant to 25,000 μg/ml. The 'B' strain in isolation falls short of this, being apparently fully resistant to 1000 μg/ml and partially to 10,000 μg/ml.

The experiment, though crude, shows that to obtain resistant mutants, upon whose drug-fastness we can rely, isolated colonies are essential, and probably the ideal count lies between 10 and 100, as too small a number on the other hand will introduce other errors.

2. DETERIORATION OF ANTIBIOTIC

A somewhat similar phenomenon of artificially high colony counts occurs where antibiotic plates are not freshly prepared or where experiments are unduly prolonged. Streptomycin is heat-labile especially above 60°C, and 60% is lost e.g. during the inspissation of Loewenstein-Jensen medium. The loss is, however, very much smaller at 37°C, and for periods of a few days, such as we are using, are largely offset by the alkalinity of the MacConkey Agar, for streptomycin is twelve times more potent at pH 8 than at pH 6.5. However if the cultures of any of the previous experiments are incubated for any longer period there will be a combined loss of drug by deterioration and also by absorption by the growing resistant colonies.

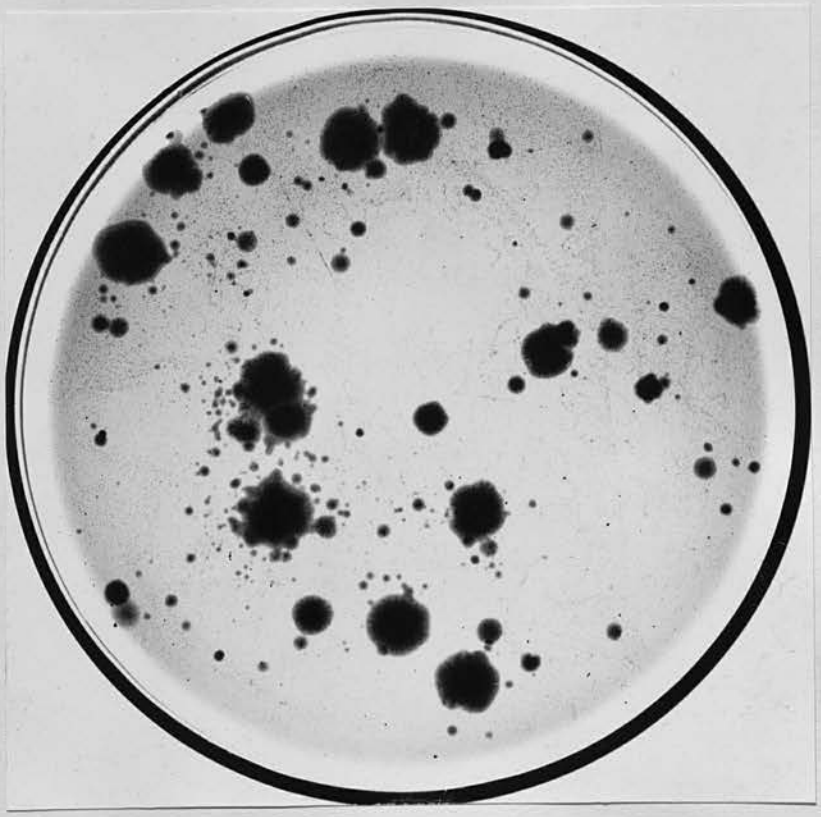


Fig: 36. *The underlying mechanism of the phenomenon seen in Fig. 34.*

Satellites of low drug-resistance are growing round each large highly resistant colony.

EXPERIMENT 34.

Fig: 36 shows the result of keeping a 1000 μg/ml streptomycin - containing plate from Experiment 5 showing 13 resistant mutants for a week at 37°C, and then photographing the resulting growth by transmitted light. It will be seen that there are still the 13 original true mutant colonies now grown to giant size. But scattered over the medium are fully a hundred much smaller colonies, generally less than a tenth the size. They are particularly numerous in the area where 3 of the large mutants lie closely together and one may speculate whether such "satellitism"implies that the area has been denuded of streptomycin thus enabling weaker bacilli to grow. There must also be the effect of time on the lability of the streptomycin itself, and elsewhere on the medium this factor must presumably predominate.

EXPERIMENT 35.

To confirm that the main colony "M" is a true 'First Day Mutant', and that the small satellite colonies "S" appearing around the sixth day in the vicinity are, as it were, opportunists, subcultures were prepared as follows.

Overnight cultures in broth containing 80 μg/ml. streptomycin were made of one of the giant colonies from Experiment 16, and from one of the minor colonies adjacent

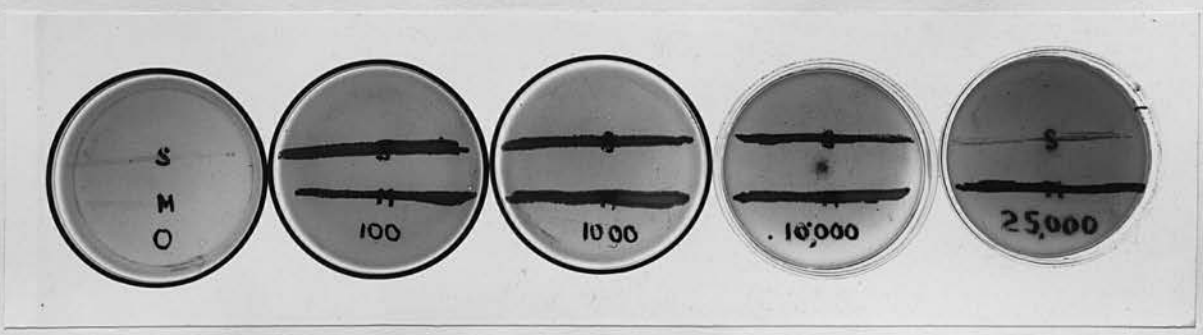


Fig: 37. *Further confirmation of the explanation advanced in Fig: 35. Streak sub-cultures have been made from the large main colonies ('M') and from the satellite colonies ('S'). Incubation of the streaks on agar plates containing 0; 100; 1,000; 10,000; and 25,000 μg./ml. streptomycin shows that their viabilities are quite different. 'M' is truly streptomycin-dependant and highly resistant (to 25,000 μg.), while 'S' is only streptomycin-dependant and moderately resistant (to 10,000 μg./ml. streptomycin).*

but discrete. These were carefully adjusted to the same turbidity (Brown No. 4) and streaked in parallel on 5 MacConkey Agar plates containing respectively 0; 100; 1,000; 10,000; and 25,000 μg/ml. of streptomycin. After overnight incubation at 37°C the results are seen in Fig: 37, photographed by transmitted light. Both clones are again truly streptomycin-dependant and resistant; but while the main colony "M" grows best at 25,000 μg, diminishing with lower strengths of drug, the satellite "S" grows best at 100 μg diminishing at higher strengths.

The conclusion is once again that heavy inoculation of antibiotic plates gives falsely high resistant counts as weak mutants are presumably helped by strong ones using up the available drug.

3. SIMULTANEOUS DIVERSE MUTATIONS

A further complication which tends to be overlooked when pre-occupied with the single phenomenon of drug-resistance is that this mutation, or rather group of mutations, is only one facet of a whole host of mutations affecting morphological characters such as pigmentation, virulence, nutrition and biochemical properties. If one assumes that these phenomena also are subject to variation of all degrees and in different directions simultaneously, it is possible to realise the

enormous diversity to which even simple bacteria could evolve.

For our present purposes only the commonest co-existant mutations are likely to obtrude. And perhaps the commonest is 'S' to 'R' type of colony associated with loss of virulence, and, in the case of E. coli such as we have been using, producing the distinctive traditional rough colonies from smooth. With Salm. typhi-murium these mutants comprise some 2% of the population, and for E. coli they seem quite as common (See Fig: 38).

4. NON-MUTATIONAL MULTIPLE DRUG-RESISTANCE

Mention must also be made of a rare phenomenon, multiple drug-resistance which has appeared as a single transmissable characteristic in Japan [458]. The explanation is that certain Escherichia possess "Resistance Transfer Factor" or R.T.F., which they convey by transduction to other Escherichia and even to Shigella, making them resistant to e.g. streptomycin, tetracyclines, chloramphenicol, and sulphonamides. Although R.T.F. has a frequency of about 1 in 10^7 it is not a mutation, and the streptomycin-resistance it confers is of low degree never exceeding 25 μg./ml. It is ineffective in bile, as in our MacConkey's media, and is more readily conveyed in the lag phase than in our rapidly

Fig: 38. *Rough and smooth colonies of the strain E. coli, N.T.C. No: 86, seen on MacConkey Agar. Although itself a mutation, it is not to be confused with the variation in size on antibiotic-containing plates, seen in Fig: 35, due to the different rates of growth which results from drug-resistance probably by adaptation as well as by mutation.*

multiplying bacilli. For these reasons we have felt able to disregard the factor in our experiments.

APPLICATION OF MUTATION RATE EXPERIMENTS TO MYCO. TUBERCULOSIS

It appears from the foregoing experiments with E. coli that resistance to streptomycin arises from the cumulative effect of mutations which occur so constantly that their numbers can be predicted. For practical purposes it is enough to know that the regularity of such mutations in any strain would justify deductions based on a suitable laboratory test. The "Slope Diffusion" sensitivity test for Myco. tuberculosis described in the next Chapter affords a means of providing gradients of potency of streptomycin so that colonies of tubercle bacilli of varying resistance can appear and be counted.

By analogy with the results seen in the E. coli experiments it is probably the subsequent multiplying potentialities of the common low-resistance colonies which are the hazard, not the isolated highly resistant one which they so vastly outnumber. For example, having examined the cultures from a case of tuberculosis after four weeks' incubation for zones of inhibition broadly indicating the sensitivity of the bacilli, a search is made with a hand-lens for minute single colonies within the zone, if necessary the vials being re-incubated for a further fortnight to help their

recognition. The onset of widespread resistance in the strain is foretold by the recession of the inhibited zones or by a scatter of resistant colonies within these zones.

To be of clinical value the results of such a test should, of course, be available prior to, or at least at an early stage in, drug treatment, and the "Slope Diffusion" test on the first sub-culture will often give a just visible zone after only one month from the taking of the specimen.

PART III.

DRUG-RESISTANT TUBERCULOSIS

AS A "NEW DISEASE"

CHAPTER 10. THE GROWING PREVALENCE OF DRUG-RESISTANCE AMONG TUBERCLE BACILLI; AND ITS ASSESSMENT

In contrast to the experience with staphylococci, in which resistance to antibiotics is generally attributable to infection from a nasal carrier with an already drug-fast strain [168, 171], possibly even ones of natural occurrence [125], the problem in the chemotherapy of tuberculosis has been quite different up to the present time. Strains of tubercle bacilli, in the great majority of cases, have been initially sensitive to such drugs as streptomycin, p-amino-salicylic acid, and isoniazid; but have tended to acquire resistance while undergoing treatment [305], and evidence is accumulating that the change is a lasting one [433].

Clinical relapses after an initially favourable response to streptomycin have been recognised as a major hazard in the treatment of pulmonary tuberculosis [300], and tuberculous meningitis [386]. Such relapses are most usual in pulmonary tuberculosis during the second and third month [138] and can be correlated with the more or less abrupt emergence of resistant strains of bacilli in the cultures submitted to streptomycin-sensitivity tests. In tuberculous meningitis, however, proved cases of *in vitro* resistance are rarer [381].

No doubt some of these resistant cases date back to the era of single-drug therapy; but expectations that combinations of drugs would completely eradicate the risk appear to have proved unduly sanguine. There are well-authenticated cases of meticulously conducted courses of multiple drug therapy merely inducing resistance [233]. It seems clear that the need for periodical drug sensitivity tests on every case will increase rather than diminish even with combined drug-therapy, and with a lengthening list of drugs there is urgent need for a drastic simplification of method.

But in addition there is the likelihood that cases of tuberculosis, which show initial resistance to streptomycin, will also become increasingly prevalent, and this emphasises the need for routine sensitivity tests to include new cases. To be of value the results of any sensitivity tests should, of course, be available prior to, or, at least, at an early stage in streptomycin treatment, and the ideal plan would seem to be to test the diagnostic cultures of cases on entry to hospitals or sanatoria, and to record the findings for reference. Moreover recent research has indicated that it may well be possible by combining streptomycin with some other therapy, such as p-aminosalicylic acid [302] or the sulphones [250], to reduce the chances of resistant variants developing. The "Slope Diffusion" method, which will be described, offers a

simple and rapid test suitable for routine use in small laboratories, and by giving an indication of the occurrence of resistant variants, as well as of the general level of sensitivity, the test may suggest the probable response to drugs.

It is generally accepted that the onset of drug resistance has been due to the multiplication of rare naturally occurring resistant bacilli, which supersede the original sensitive majority of bacilli as the latter are eliminated [81, 127, 496, 497] (Fig: 39).

Early identification of these rare resistant bacilli is important as drug combinations can retard their emergence [302]. The rationale of these measures is based on the likelihood that bacilli which possess more than one such natural drug insusceptibility will be in still greater rarity; but double or even triple chemotherapy, to be effective, must be given before any such resistant strain develops predominance, for once this occurs to one drug its admixture confers no advantage over the alternative drug given alone.

SENSITIVITY TESTS CURRENTLY IN USE

The M.R.C. - Dubos Method

This method [301] makes use of visible turbidity in the liquid culture medium of Dubos as an index of growth. To wet

Fig: 39. *A single large resistant colony of Myco. tuberculosis strain H37 Rv surviving on a Lowenstein-Jensen slope with 100 µg./ml. of streptomycin at the base.*

the lipoid cell-membrane of the tubercle bacillus [128], and give a submerged growth [79], sorbiton monodeate ("Tween 80") is added [105], and to offset the antagonistic effect of this detergent [498] the medium is enriched with bovine serum albumin [90, 103, 382]. That the Tween 80 is itself bacteriostatic can hardly be doubted [102, 116]. A Ziehl-Neelsen film of a Dubos culture shows that many of the bacilli have lost their acid-fastness in whole or in part by the action of the detergent.

The technical procedure involves culturing on solid medium, and then usually three successive subcultures in Dubos medium to obtain a more and more diffuse growth. The third subculture is distributed in eight bottles containing doubling dilutions of streptomycin or other drug. and the minimum amount of the drug required to inhibit completely this diffuse growth, and give a clear solution, is determined. The stock culture H37Rv is tested in parallel as a control.

Unfortunately the method is impracticable for small laboratories as it may take two months and some three successive cultures to obtain the turbidity used as an index of growth, and at each subculture there is a risk of contamination, and not least of danger to the worker. However the test is quantitatively most precise, and when required, for example for evaluating a new drug, time and

even a subculture can sometimes be saved by shaking the cultures daily. For this purpose a wooden board drilled with $\frac{7}{8}$" holes an inch deep to hold bijou bottles may be made to fit the Kahn shaker. Each bijou contains two sterile 5 mm beads in Dubos medium, and thirty minutes shaking gives a suspension which is incubated as the first subculture. The boards can be stacked in the incubator and hold the bottles sufficiently firmly to allow daily rocking by hand to accelerate further diffuse growth.

But a much more serious objection is that tests in liquid media give no indication of mixed sensitive and resistant bacilli and their relative numbers. Crofton and Mitchison [81] by testing individual colonies from the same culture from a case under treatment have shown that there may be different degrees of resistance present. It is therefore essential to make the inoculum for any test truly representative by drawing the loop over a wide area of the primary culture. But even if we are satisfied with our random sampling the method only gives an overall picture of the sensitiveness or resistance of the patient's strain at the time of the test, and no indication of its future state. It could not, for instance, discriminate between a large number of moderately resistant bacilli partially inhibited and multiplying slowly, and a few highly resistant bacilli totally uninhibited and multiplying fast.

Fig: 40. *Holt and Cruikshank's method of showing drug-resistance in Myco. tuberculosis.*
A Lowenstein-Jensen slope with 100 µg./ml. of streptomycin incorporated before inspissation is shown on which 13 resistant colonies are growing.

Fig: 41. *Another possible method of showing drug-resistance in Myco. tuberculosis. A Lowenstein-Jensen plate with 100 µg./ml. of streptomycin in the gutter has been inoculated with 3 strains - reading downwards, sensitive, moderately sensitive and resistant.*

Only a method employing isolation on solid culture media can hope to isolate the different colonies which may be present together in a single patient's strain (Figs: 40 and 41).

Karlson & Needham's Method [229]

In this American method egg-nutrient agar had 1, 10 & 100 μg/ml. of streptomycin added at 50°C and subculture gave a broad indication of resistant bacilli.

Holt & Cruikshank's Method [200]

Subcultures are made on Lowenstein-Jensen medium in which drugs in various strengths have been included, and the lowest concentrations giving complete inhibition, or not more than 20 colonies, are usually accepted as effective. The streptomycin can be added either by mixing with the medium before its solidification, with allowance for 60% loss of potency on heating, or by allowing the solidified slope to absorb streptomycin solution uniformly over its surface while lying horizontally.

Tests employing slopes, such as these, reduce the risk inseparable from the use of liquid medium, of resistant bacilli which may have been comparatively scanty in the inoculum multiplying to dominate the final result. But there is still the impossibility of distinguishing between inhibited growth and inadequate or even absent inoculum, and the choosing of an end-point of say 20 colonies is somewhat arbitrary, for

these 20 resistant colonies may, as previously explained, be the very ones in which we are interested - the progenitors of a future resistant strain. Nevertheless the methods are very convenient especially for the non-antibiotic drugs isoniazid and P.A.S. Tubercle bacilli resistant to these drugs appear to be equally resistant *in vitro* to "Nupasal-213" (o-hydroxybenzal isonicotinyl hydrazone) and "Dipasic" (isoniazid-p-aminosalicylate) respectively, as the following experiment shows:-

Resistance to Isoniazid, P.A.S., and their derivatives

Six strains partially, and seven wholly resistant to isoniazid were found to show identical degrees of resistance to "Nupasal-213". Five strengths of each drug were used - 0.2, 1, 5, 10, and 50 μg. per ml.

Fifteen strains resistant to P.A.S., isoniazid, or both were tested similarly against P.A.S., isoniazid, and "Dipasic" (a drug combining the other two). Eight sensitive to P.A.S. were also sensitive to "Dipasic". Four partially, and one wholly resistant to P.A.S. were also resistant to "Dipasic" to a comparable degree. The two remaining strains in their greater resistance to "Dipasic" than to P.A.S. apparently reflected the influence of isoniazid to which they were wholly resistant.

This suggests that strains of tubercle bacilli resistant in vitro to isoniazid show identical resistance to "Nupasal-213", and that strains resistant to P.A.S., and/or isonizid will also be resistant to "Dipasic". Additional tests with these proprietory drugs do not, therefore, seem justified [71, 108, 243, 341, 432, 433, 457].

THE SLOPE DIFFUSION METHOD

The Slope Diffusion Test introduced twelve years ago[437] has been found to give consistent results, and has several advantages in the modified form now described [435]. It depends upon the fact that if the antibiotics, e.g. streptomycin or viomycin are placed in suitable strength at the foot of a Lowenstein-Jensen slopw, diffusion will occur for up to two inches. There appears to be a gradient in this diffusion diminishing towards the neck of the vial so that some visible growth of Myco. tuberculosis can survive to define its limits after about three weeks. That it is diffusion and not capillary spread is suggested by the phenomenon occurring equally well when the bottles lie flat. For the same reason the originally suggested name of "Vertical Diffusion Test" is inappropriate.

Such a short gradient of diffusion occurs only with the macro-molecular compounds such as the sulphates or chlorides

TABLE 33

MOLECULAR WEIGHTS OF TUBERCULOSTATIC DRUGS

Drug	Mol. wt.
Iso-niazid	145
P.A.S.	153
Na. P.A.S.	211
Ca P.A.S.	380
Streptomycin hydrochloride	691
Streptomycin sulphate	1457
Streptomycin $CaCl_2$ complex	1493
Dihydro streptomycin chloride	693
Dihydro " sulphate	1461
Viomycin base	503
Viomycin sulphate	1101
Oxytetracycline hydrochloride	489
Cycloserine base	733
Fusidid acid	516

of antibiotics, whose molecular weights are between 500-1500 (See Table 33). The method is impracticable in small vials for e.g. p-aminosalicylic acid (P.A.S.), isoniazid (I.N.H.) and pyrazinamide (P.Z.A.), presumably because their molecular weights of about 150 are so small that rapid diffusion occurs throughout the whole slope. For these the usual media with the drugs incorporated may be retained, in the case of pyrazinamide, incidently, acidification to pH 5 being necessary to ensure full potency. Alternatively a modified test using specially long tubes may be employed as described below.

Sensitivity to oxytetracycline and cycloserine can be tested by the Slope Diffusion Method; but as these drugs in the usual strengths are only tuberculostatic, not bactericidal, freshly made slopes should be used and read as soon as growth is apparent to the naked eye. Cycloserine is also very unstable in neutral or acid medium; but in practice the egg slopes are sufficiently alkaline. Kanamycin and Fucidin (Fusidic acid) have also given reliable tuberculostatic zones on diffusion slopes.

Methods

Lowenstein-Jensen slopes are prepared in universal containers or 1 oz. screw-capped vials, and the condensation water is poured off before adding to the foot of the slope

Fig: 42. *The Slope Diffusion Test for the Streptomycin-sensitivity of Myco. tuberculosis. Culture of H37 Rv, a typical sensitive strain, showing 1" zone of inhibition above 50 µg./ml. of streptomycin, in which no resistant mutants are yet visible.*

1 ml. of a solution of 2% agar, to which streptomycin 50 μg./ml. or 200 μg./ml. pf viomycin, cycloserine or other tuberculastatic antibiotics has been added at about 60°C. The bottles are stood upright while the agar sets, and the caps marked to identify the antibiotic: they retain their potency for at least a month in the refrigerator. The greater dose of viomycin cycloserine etc. needed is due apparently to their lower strength and lesser stability than streptomycin [227, 406, 499].

For use the slope is evenly spread all over with inoculum, preferably a primary Lowenstein-Jensen culture emulsified in 1 ml. of Dubos medium, and it is then incubated for 3-4 weeks. Sensitivity is shown by the zone of inhibition extending fully half way up the slope in most cases initially but receding with subsequent specimens as resistance develops. The stock culture H37Rv is grown as a control with each batch of tests as a typical sensitive strain (Fig: 42). It is important to use full 1 oz. vials. Any smaller size will not display the residual growth which contrasts with the inhibited lower zone. Moreover tubercle bacilli being aerobes small bottles probably discourage growth.

The total time required for a sensitively test by this method is 6-7 weeks; but where urgent reporting is paramount it has been found practicable to duplicate the diagnostic cultures with these diffusion slopes inoculated

directly with sputum after concentration, although the results are not, of course, so clear cut as with an evenly spread subculture.

METHYLENE BLUE MEDIUM FOR DISTINGUISHING VIRULENCE

Animal inoculation, the most generally reliable test of virulence in suspected tubercle bacilli [367], is open to fallacies from isoniazid-resistant organisms which while increasingly responsible for human tuberculosis [435] can be of low pathogenicity to guinea-pigs and mice [74, 228]. Alternative tests of virulence include "cording" of bacilli in cultures [97] and their ability to absorb dyes in solution [96, 104, 207], but inconsistencies occur from mixed strains [189] which can only be separated by isolation on solid media.

In spite of reports of its toxicity [333, 440], methylene blue can replace the malachite green in Lowenstein Jensen and will detect the dehydrogenase of virulent strains [30, 192, 194]. If 2 cc. of 1% aqueous methylene blue are added to each 100 cc. of medium, and the slopes kept in air-tight vials to prevent later re-oxidation, virulent tubercle bacilli including those resistant to isoniazid will stain dark blue while saprophytic mycobacteria and gram negative contaminants remain colourless [436].

STREPTOMYCIN-ANTAGONIST MEDIUM

Where the patient has already been on streptomycin therapy, culture of the infecting organism is usually unsuccessful unless a streptomycin-antagonist[10,12,100,121,144 264,353,365,366,373,374,421], is added to the medium. We employ Lowenstein-Jensen slopes in which 2 mg. cysteine hydrochloride per ml. of the medium is added before pouring. During subsequent heating the powerful reducing action of the cysteine may bleach the malachite green but on standing with the bottle caps screwed down the dye becomes more blue than before. When growth has been achieved on the cysteine medium the organisms are inoculated onto the streptomycin-containing bottles for subsequent sensitivity tests. Using this method, one specimen from a case which had had nine months' treatment was successfully grown after five abortive attempts on ordinary medium.

Drug Concentrations on Slope Diffusion Media

All the tuberculostatic drugs used in the Slope Diffusion test are crystalloids, and diffuse readily from the agar at the foot of the slope. Presumably the subsequent spread is up the surface of the egg medium; penetration is unlikely to any appreciable extent as the egg lattice is already saturated with water.

Estimation of drug-loss on gradient

By using a sterile cork borer plugs were punched out at ¼" intervals up the slopes which had been stored in the refrigerator for a week. These were placed on agar plates and time allowed for diffusion before inoculating with the test organism, Esch. coli N.T.C. 1094. It was found that 50 μg. streptomycin had dropped to about 10 g. at ¾", the estimate being obtained by a comparison of inhititory zones with those from plugs containing known amounts of drug. This ¾" up the slope is the usual point where equilibrium is reached between a growing strain of sensitive Myco. tuberculosis and the diffusing antibiotic.

Loss of potency of streptomycin during incubation

This is the other possible variable affecting the antibiotic during the test, and biological assays were therefore made of streptomycin kept at 37°C as follows:-

Test organism: Klebsiella pneumoniae sensitive to 1 μg./ml. streptomycin in glucose broth.

Test concentration of drug: Streptomycin hydrochloride 16 μg./ml. in water.

7	days at 37°C	16 μg./ml.
14	" " "	16 "
21	" " "	8 "
28	" " "	8 "

It was concluded that there would be no appreciable loss from this cause during the first fortnight of the test.

THE LONG-SLOPE DIFFUSION TEST

Schmiedal [384] and later Kunz and Rudnick [247] showed that similar diffusion zones do in fact occur with the drugs isoniazid and p-aminosalicylic acid; but that they are too extensive to be visible within the confines of a 1-oz. bottle. Using 30 μg. of I.N.H. or 100 μg. of P.A.S. in 1 ml. agar at the foot of a Lowenstein-Jensen slope in a sufficiently tall container such as a 7 x $\frac{7}{8}$" boiling tube with a rubber stopper, zones of inhibition of some 10 cm. are seen with "fully sensitive" strains, (but see Chapter 6, page concerning P.A.S. and Figs: 43 and 44). We use specially made * 6 x 11/16" screw-capped test-tubes for these drugs, and also for ethionamide, 100 μg. of which gives a comparable 10 cm. zone. Pyrazinamide, even in a concentration of 10 mg., was ineffective. The demonstration of heterogeneity in the strain and especially of resistant mutants is seen with these drugs as with the antibiotics, and this further advantage is described in the next Chapter.

* Obtainable from Johnsen & Jorgensen, Trident Glass Works Herringham Road, Charlton, London, S.E.7.

Fig: 43. *Schmiedel's [384] Long Slope modification of the Slope Diffusion Test. Using tubes 6" long the method can be used for isoniazid (33 µg./ml. at foot of tube), as seen in the three left-hand examples, and P.A.S. (100 µg./ml.), as seen in the two right-hand tubes.*

Fig: 44. *A close-up photograph of Fig: 43. Note that while the inhibitory zones for isoniazid are clear-cut. those for P.A.S. apparently show three steps of (a) confluent growth at top, (b) semi-confluent growth in the mid-tube, and (c) scanty colonies below.*

Tsukamura [443] identified two P.A.S.-resistant genotypes:-

(i) Resistant to 1-2 μg., with a frequency of about 2 per 100,000.

(ii) Resistant to 5-100 μg., with a frequency of about 1 per 100 million.

The three steps could, therefore, be consistent with this interpretation:-

(a) Unrestricted growth due to sub-minimal drug.

(b) Growth restricted to the two resistant genotypes.

(c) Growth restricted to the highly resistant genotype only.

Results of Slope Diffusion Sensitivity Test

Tables 34 (first line) and 35 show the results from 100 newly isolated tuberculous cases for the culture's sensitivity to streptomycin and viomycin respectively, while Table 36 is the suggested interpretation for these and other tuberculostatic drugs. In the case of streptomycin a zone of inhibition of $\frac{3}{4}$" or more indicates average sensitivity, while less than $\frac{1}{4}$" implies resistance, and it will be seen from Table 37 that there is good correlation between these readings and those given by media incorporating the drug. The Slope Diffusion method has the advantage that the surviving growth in the upper part of the bottle confirms that inoculation and incubation have been adequate for the test; but of greater importance is the overall diminution of the zone of inhibition which is proportionate to the increase of drug-resistance. This is seen in Table 34 where tests were made in series some years ago, after successive months of the single drug therapy then in vogue with streptomycin. But over and above all this the Slope Diffusion Test lends itself to our present studies, because the gradient provided for the concentration of the drug is especially conducive to the emergence of resistant mutants of widely varying degree (Fig: 45).

Fig: 45. *Slope Diffusion Tests with streptomycin against strains of Myco. tuberculosis.*

Left: A resistant culture due, in this case, to numerous mutant colonies of different degrees of drug-resistance.

Right: A partially sensitive culture. There is $\frac{3}{4}$" inhibited zone above 50 μg./ml. of streptomycin; but this has a margin of weakly resistant mutants giving a "frayed edge", and there are in addition 2 highly resistant mutants near the base on the right.

TABLE 34

RESULTS OF SLOPE DIFFUSION SENSITIVITY TESTS ON STRAINS OF MYCO. TUBERCULOSIS WITH 50 µg. STREPTOMYCIN

Months of treatment with streptomycin.	Resistant ($< \frac{1}{4}$" inhibition)	Moderately sensitive ($\frac{1}{4}$"-$\frac{3}{4}$" inhibition)	Sensitive ($\frac{3}{4}$"-$1\frac{1}{2}$" inhibition)	Very sensitive ($> 1\frac{1}{2}$" inhibition)	Total
0	3	13	32	17	65
1	1	6	8	3	18
2	1	5	11	-	17
					100

TABLE 35

RESULTS OF SLOPE DIFFUSION SENSITIVITY TESTS ON STRAINS OF MYCO. TUBERCULOSIS WITH 250 µg. VIOMYCIN. UNTREATED CASES.

Resistant ($< \frac{1}{4}$" inhibition)	Moderately sensitive ($\frac{1}{4}$"-$\frac{3}{4}$" inhibition)	Sensitive ($\frac{3}{4}$"-$1\frac{1}{2}$" inhibition)	Very sensitive ($> 1\frac{1}{2}$" inhibition)	Total
-	61	34	5	100

TABLE 36

SLOPE DIFFUSION TEST

SUGGESTED INTERPRETATION OF ZONES.

(Read after 3 weeks' incubation).

Drug	*Conc. in 1 ml. agar in μg.*	*Zone of inhibn.*	*Interptn.*
P.A.S.	*100*	$\frac{1}{4}$"	$S^{\pm}_{-}$
		$\frac{1}{4}$ - $2\frac{1}{2}$"	$S^{\pm}_{-}$
		$2\frac{1}{2}$+"	*S*
I.N.H.	*33*	$1\frac{1}{2}$"	$S^{\pm}_{-}$
		2+"	*S*
Streptomycin	*50*		
Viomycin *Terramycin* *Cycloserine* *Kanamycin*	*200*	$\frac{1}{4}$ - $\frac{3}{4}$"	$S^{\pm}_{-}$
		$\frac{3}{4}$+"	*S*
Ethionamide	*100*	1+"	*S*

S = Senstitve, $S^{\pm}_{-}$ *= moderately senstitve, t.e. becoming resistant.*

$S^{\pm}_{-}$ *= only slightly sensitive, t.e. resistance imminent.*

TABLE 37

COMPARISON OF STREPTOMYCIN SENSITIVITY TESTS ON 100 UNTREATED CASES BY THE METHODS OF SLOPE DIFFUSION AND INCORPORATED SLOPE (3 μg./ml. STREPTOMYCIN).

Resistant by both tests (under $\frac{1}{4}$" and over 20 colonies respectively).	9
Sensitive by both tests (over $\frac{3}{4}$" and no colonies).	75
Moderately sensitive by both tests ($\frac{1}{4}$"-$\frac{3}{4}$" and under 20 colonies).	8
Moderately sensitive by diffusion, sensitive by incorporated slope.	6
Sensitive by diffusion, moderately sensitive by incorporated slope.	2
Contradictory results (resistant/sensitive or sensitive/resistant).	0

TABLE 38

INITIAL SENSITIVITY TO STREPTOMYCIN OF 58 STRAINS OF MYCO. TUBERCULOSIS ISOLATED BETWEEN 1948 AND 1950

Concentration of streptomycin in Dubos medium (μg./ml.)	*4+*	*4*	*2*	*1*	*½*	*¼*	*⅛*	*1/16*
Number of sensitive strains.	*1*	*1*	*6*	*13*	*12*	*18*	*4*	*3*

TABLE 39

INCIDENCE IN GLASGOW DURING 1955-1956 OF RESISTANCE TO FOUR TUBERCULOSTATIC DRUGS

	Newly diagnosed cases prior to treatment	*Hospital cases previously treated*	
Sensitive to I.N.H., streptomycin, P.A.S. and viomycin	*106*	*126*	
Resistant to:-			
I.N.H.	*1*	*21*	@
Streptomycin	*9*	*55*	
P.A.S.	*1*	*3*	
Viomycin	-	-	
I.N.H. and streptomycin	-	*30*	
I.N.H. and P.A.S.	-	*1*	*
I.N.H. and viomycin	-	-	
Streptomycin and P.A.S.	-	*6*	
Streptomycin and viomycin	-	*4*	
P.A.S. and viomycin	-	-	
I.N.H., streptomycin and P.A.S.	-	*8*	
I.N.H., streptomycin and viomycin	-	*7*	
P.A.S., streptomycin and viomycin	-	*1*	
P.A.S., I.N.H. and viomycin	-	-	
Streptomycin, I.N.H., P.A.S. and viomycin	-	*1*	
	117	*263*	

@ *Also resistant to "Nupasal-213" in 13 cases tested.*

* *Also resistant to "Dipasic".*

USE OF SERIAL SLOPE DIFFUSION TESTS FOR STUDYING STREPTOMYCIN-RESISTANT MUTATIONS

Welch [461] has suggested that the real significance of persistent growth of organisms after the administration of drugs is that it portends the coming of drug-resistance. In tuberculosis one can go further and say that the most significant feature of in vitro tests during chemotherapy is that there is growth at all - a fact which tends to be overlooked. The persistance of tubercle bacilli after treatment with streptomycin 1 gm. daily was shown by us in the following series in 1960:-

Cultures were made from pooled three early-morning sputa from 100 cases, and 36 yielded no growth at all.

Prior to treatment 64 strains grew and were sensitive.
After 1 month 27 of these still grew and were sensitive.
" 2 months 7 " " " " " " "
" 3 " 3 " " " " " " "
" 4 " 1 " " " " " " "
" 5 " 1 " " " " " " "

Originally strains of Myco. tuberculosis showed little variation in the degree of streptomycin-sensitivity. Thus of 58 strains measured in Dubos medium between 1948 - 1950, 74%

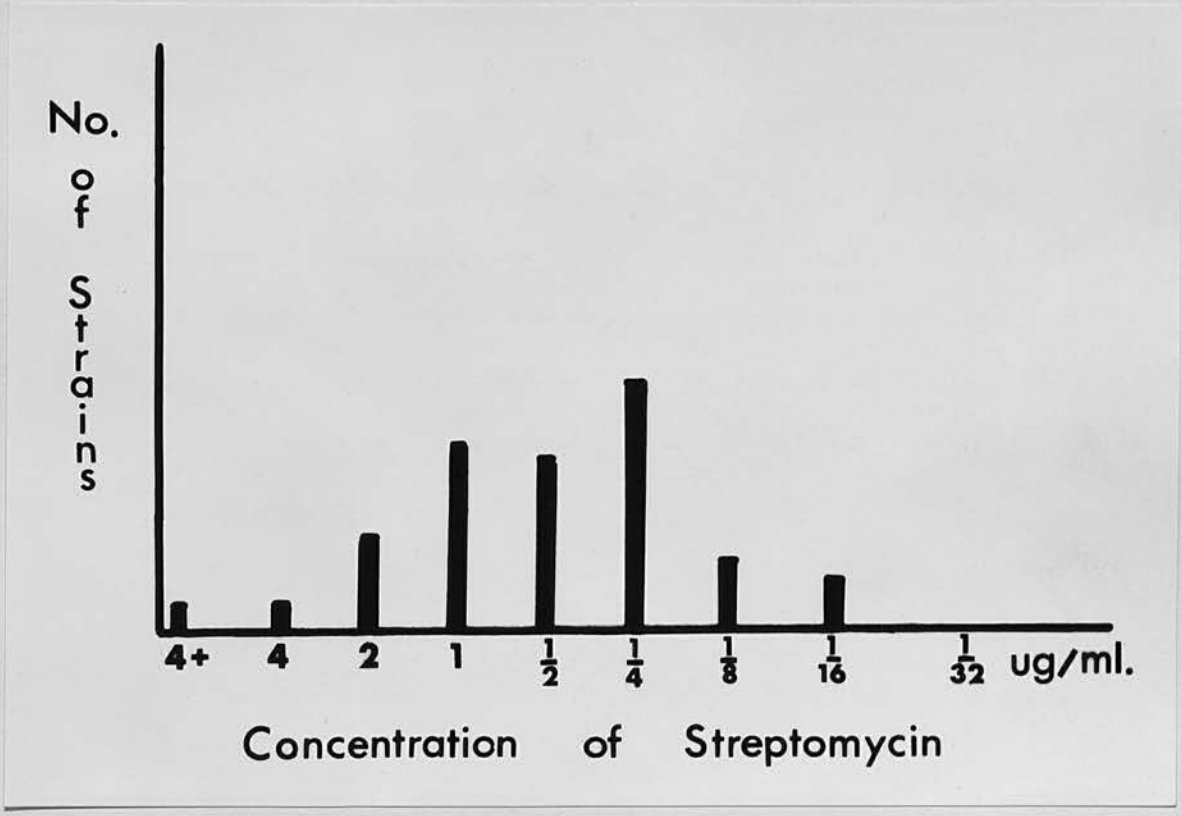
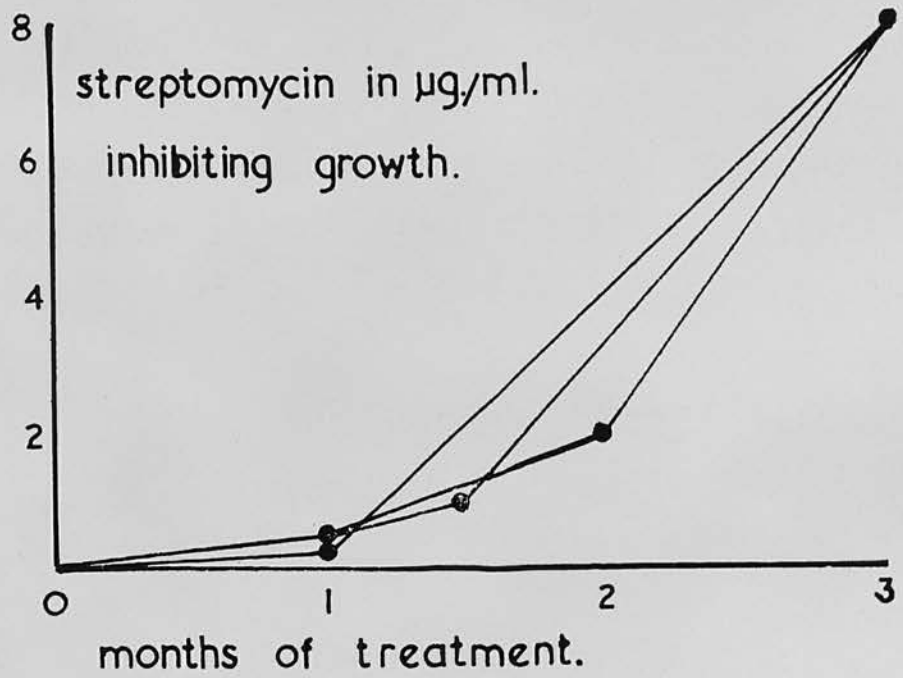


Fig: 46. *The Frequency Distribution of the initial sensitivity to streptomycin of the 58 strains of Myco. tuberculosis recorded in Table 38. Note the almost symmetrical curve around the value of $\frac{1}{2}$ µg./ml.*

were sensitive to between 1 and $\frac{1}{4}$ μg/ml. (Table 38 and Fig: 46), while subsequent progress under the single-drug therapy of this period revealed a steepening curve of streptomycin resistance, as shown by the graph of three cases (Fig: 47). The "single-step" rises seen here are in marked contrast with the more familiar "multi-steps" of penicillin resistance, and are, of course, the outcome of the cumulative mutations occurring with streptomycin which we have described in the earlier Chapters (Part 2).

Initial resistance to a tuberculostatic drug may occur spontaneously; but this is rare. More commonly it is the outcome of therapy, either in the patient himself or by infection from a treated case [82]. That this is no inconsiderable risk in a populous area, especially with streptomycin and isoniazid, is shown by Table 39. The implications of these several varieties of mutant resistance will be discussed more fully in the next Chapter.

The particular merit of the Slope Diffusion Test for our present intention is that, affording as it does for all practical purposes an almost infinite gradient of streptomycin, mutants of varying resistance will appear, and can be studied with a lens as they do so between the fourth and sixth week of incubation. Occasionally a lack of the customary abruptness between growth and inhibition indicates insusceptible



Increasing resistance to streptomycin in Dubos medium of three strains of *Myco. tuberculosis* following streptomycin treatment.

Fig: 47. The so-called "Single-step" curve characterising the onset of streptomycin-resistance, which contrasts with the more gradual "Multi-step" pattern seen with penicillin.

clones of low degree of resistance being sufficiently numerous to portend imminent general resistance; but more commonly there are isolated resistant colonies within the zone of general inhibition as seen in the illustration (Fig. 44). Drug-containing media will also be used in the studies which follow; but supplementary, and aimed at isolating these mutant colonies - the harbingers of resistant populations of tubercle bacilli.

CHAPTER 11. THE GENESIS OF AN EPIDEMIC DISEASE - DRUG-RESISTANT TUBERCULOSIS

The Source of Resistant Tubercle Bacilli

NATURALLY RESISTANT STRAINS OF MYCOBACTERIA

Mention was made in Chapter 6 of the suggestion of Fairbrother [125] that penicillin-resistant staphylococci were in fact the primitive type, and that the emergence of such drug-resistance now was in fact merely a reversion to the original saprophyte by the more specialised, and therefore penicillin-sensitive, modern parasite. Staphylococci found since in the wilds of New Guinea [375], which could not possibly have encountered penicillin therapy, and yet are penicillin-resistant, appear to bear this out.

One inevitably speculates whether a similar phenomenon is in fact operating with resistant tubercle bacilli, and in looking for naturally drug-resistant strains one is confronted by a curious group of Mycobacteria occupying the borderland between saprophytism and potential pathogenicity. These are the " Atypical Mycobacteria" which appear sporadically, and of which we have found t e n examples over a decade, all at least in association with infections. All had apparently co-existed in the body with Mycobacterium tuberculosis, or had themselves

been implicated in chronic insidious diseases strangely reminiscent of the protean manifestations of tuberculosis, though of far lower virulence.

THE ANONYMOUS MYCOBACTERIA AND THEIR RELATION TO THE TUBERCLE BACILLUS

During the last ten years this group of so-called "Atypical Tubercle Bacilli" have been recognised repeatedly in association with human disease. Runyon [190, 379], classified them into four groups:-

I Photochromogens. Yellow or red on exposure to light.

II Scotochromogens. Similarly coloured even in the dark.

III Uncoloured pleomorphic 'Battey Strains'.

IV Rapid growers, even at room temperature, and uncoloured.

All are relatively drug-resistant, in fact it is this feature rather than abnormal morphology or the colour of the colonies which has attracted attention to them [381]. Group I resist 1 μg. I.N.H., and 10 μg. P.A.S., while the other groups are still more resistant especially to isoniazid. Besides this, all four groups are non-pathogenic to guinea-pigs, but they may be virulent to mice, though we have not found them so, and this additional feature of experimental avirulence they share with isoniazid-resistant strains of undoubted Myco. tuberculosis from cases of human disease. Some have supposed therefore that these newly discovered Anonymous

Mycobacteria evolved from tubercle bacilli as a result of drug treatment [474], particularly as the chronic lesions in which they were found, and their situation, e.g. in the lungs and neck glands, would be regarded otherwise as typical for classical tuberculosis. The following ten cases illustrate this:-

JAMES ALLISON

Pneumoconiosis

This man had been on a 40% pneumoconiosis pension for 12 years, and in May 1962 a new opacity was noted in his chest X-rays in the left upper lobe.

He was admitted for investigation. Repeated sputum examinations for tubercle were all negative. The question of a bronchial carcinoma was raised, especially as the hilar glands became more prominent. Although bronchoscopy was negative a left pneumonectomy was carried out.

Discovery of Chromogenic A.F.B.

Histology (report 1674/62) confirmed the presence of advanced, simple pneumoconiosis and the lesion in the upper lobe was found to be an area of progressive massive fibrosis showing central cavitation.

Necrotic material from the centre of the cavity produced a growth of an acid fast bacillus which was found to be resistant to P.A.S. and I.N.A.H. and which became pigmented.

Subsequent Tests

The culture was found to be sensitive to Streptomycin; but resisted 10 μg P.A.S. and 50 μg I.N.H. It grew on ordinary Lowenstein-Jensen, and on L-J with 5% sodium salicylate, at 37°C appearing in 11 days. No growth occurred on nutrient agar, nor at 22°C on L-J. Growth assumed a butter yellow colour in the dark after 3 weeks. Catalase + +, Oxidase -. Classified as a Scotochromogen - i.e. Group II.

WILLIAM PEAT (Udston Hospital)

A Case of Pulmonary Fibrosis with a Chromogenic Bacillus.

History 1952 Found to have extensive pulmonary fibrosis and positive sputum. Sputum became negative until:-

1954 Sputum again positive and has remained positive ever since despite continuous treatment with P.A.S., Streptomycin and I.N.A.H.

1962 Sensitivity (Hamilton) reported as

P.A.S.	Resistant
Streptomycin)	
)	Sensitive
I.N.A.H.)	

November - Sensitivity (Hairmyres)

Streptomycin)	
)	
P.A.S.)	All resistant
)	
I.N.A.H.)	

Viomycin - Sensitive

? atypical acid fast bacillus.

Treated with Viomycin, Cycloserine and Ethionamide since.

Subsequent Tests

A pleomorphic beaded alcohol and acid-fast bacillus which did not grow on nutrient agar nor at room temperature. Eugonic growth on Lowenstein-Jensen after 15 days at 37°C and on the same medium with 5% Sod. salicylate. Colonies assumed a canary yellow after 3 weeks incubation in the dark. Catalase ++, Oxidase -. Culture resistant to P.A.S. 10 µg, I.N.H. 5 µg, and partially resistant to 100 µg. and 50 µg. respectively; but sensitive to Streptomycin.

Classified as a Scotochromogen (Group II).

DAVID CASEY

Cervical Adenitis apparently due to a Scotochromogenic Mycobacterium.

History

This 5-year old boy reported in May 1961 with a submandibular swelling. X-ray of the chest was equivocal, and it was decided to excise the swollen gland. The pus contained no acute pyogenic organisms but numerous acid-fast bacilli.

Laboratory Tests

The bacilli were Gram +, beaded, acid- and alcohol-fast but somewhat pleomorphic. Culture, which required 37°C, yielded a butter yellow growth even in the dark. Lowenstein-Jensen was preferred but some growth was obtained on nutrient agar after 3 weeks. The strain was sensitive to Streptomycin 3 μg, but resisted 50 μg isoniazid and 10^0 μg P.A.S. (It was also sensitive to viomycin, cycloserine, soframycin and erythromycin; but resistant to dipasic 5 μg, terramycin, ethionamide, penicillin, neomycin, polymyxin, and sulphanilamide.) It was Catalase and Oxidase -. Inoculated guinea-pigs showed no lesions after two months, nor did they react to old tuberculin i.d. The organisms was classified as a Group II Scotochromogen.

Subsequent History

The child was discharged after 5 months bed-rest in a sanatorium. His Mantoux Test was only weakly + to 1/100 O.T., and X-ray of the chest showed only one small apical focus.

MISS JOYCE HAY (25 years)

Pulmonary Tuberculosis

Found to have active pulmonary tuberculosis in October 1960 -

Sensitive to Streptomycin 3 μg., P.A.S. 2 μg., I.N.H. 1 μg.

Treated with all three and then Pycamycin with good response and all sputa and laryngeal swabs negative for tubercle since January 1961.

Discovery of Chromogenic A.F.B.

In March 1961 she was referred to the urologist for pain on micturition and haematuria.

A.F.B. found on culture of urine, and as she was a teacher she continued to be excluded from employment.

This culture was resistant to -

Streptomycin 30 μg., P.A.S. 100 μg., I.N.H. 50 μg.

Subsequent Tests

This high degree of overall resistance prompted further tests, and the strain was found to grow readily even on nutrient agar and at 22°C, giving after a fortnight a white growth changing to rosy pink on exposure to light. It was oxidase + and catalase ++, and was classified as a (Group I) photochromogen.

MISS DEIRDRE DUFFY

A case of Chronic Paronychia associated with a colourless atypical Mycobacterium.

History

At the age of 23 a chronic granuloma invaded the whole of the soft tissues of the terminal phalanx of the right little finger (See Fig: 48). Amputation was considered but her employment as a telephonist and her hobby as a pianist encouraged search for a remedy. There was even a suggestion that her infection was amphibian tuberculosis as she kept tortoises!

Laboratory Investigations

Direct films were negative; but culture of the tissue on both Lowenstein-Jensen and nutrient agar grew white granular colonies in 3 days at 37°C and more slowly at room temperature, which were microscopically long beaded acid and alcohol-fast bacilli. They were catalase +. oxidase -, and decolourised neutral red and methylene blue. They liquified gelatin and fermented lactose and sucrose without gas. Growth was a pellicle on broth, black hemispheres on tellurite, and crenated L.F. colonies on MacConkey Agar (see Fig: 49). The bacilli were avirulent to guinea-pigs and mice. They withstood 5% sodium salicylate, and they resisted the tuberculostatic drugs

Fig: 48. Paronychia of right little finger in the case of Deirdre Duffy. Infection was due to Myco. fortuitum.

Fig: 49. *Myco. fortuitum growing on MacConkey Agar after 3 days.*
Case of Deirdre Duffy.

streptomycin 30 μg., P.A.S. 100 μg., I.N.H. 5 μg., and also cycloserine, and pyrazinamide, besides penicillin. However in vitro sensitivity to sulphonamide and oxytetracycline enabled these drugs to be used systemically with slow resolution.

The organism was classified, because of its rapid growth, as in Runyon's Group IV, while culture on MacConkey's medium is said to distinguish Myco. fortuitum in this group.

MISS THOMASINA MUIR

History

This lady aged 67 had acne rosacea on her face, and, because of Lewandowsky's classical contention of its tuberculoid nature, a biopsy was cultured.

Laboratory Investigations

Culture on nutrient agar at room temperature gave a rugged white growth in 2 days, which consisted of beaded acid- and alcohol-fast bacilli but which were short and pleomorphic. Growth on tellurite gave black hemispheres, and on broth a pellicle, and it also occurred on medium with 5% sodium salicylate but not on MacConkey Agar. The culture was catalase + but oxidase -. it decolourised neutral red and methylene blue, and it gave no sugar fermentations and only slight liquefaction of gelatine. It was avirulent to guinea-pigs and mice. The tuberculostatic drugs streptomycin 30 μg., P.A.S. 100 μg., I.N.H. 5 μg., cycloserine and pyrazinamide were resisted as well as penicillin and sulphonamide: but the bacilli were sensitive to chloramphenicol and soframycin.

Tentative classification was in Runyon's Group III, largely because of the pleomorphic morphology.

MISS KATHLEEN MCKENZIE

Another rosacea-like rash of face and chest, which had persisted 10 years on the face but only a few months on the trunk.

Laboratory Investigations

Direct films were again negative; but culture on agar in the dark gave orange crusts in 3 days at 37°C and in a week at room temperature. The other tests resembled those on T. Muir's strain except that the bacilli formed a deposit in broth and were much more proteolytic in gelatine. Again there was no virulence to animals, and resistance to tuberculostatic drugs exceeded 30 μg., streptomycin, 100 μg. P.A.S., 10 μg. I.N.H., besides viomycin, cycloserine and ethionamide as well as penicillin and sulphonamide; but erythromycin, chloramphenicol and soframycin were again effective. The organism was classified as a scotochromogen, Runyon Group II.

GEORGE PENMAN

A case of Cervical Adenitis associated with a Photochromogen.

This 25-year old salesman had had his right cervical glands swollen for 7 weeks. Culture of the excised gland yielded buff-coloured crusts in one week on nutrient agar at room temperature which deepened to a pink colour in the light. Supplementary tests resembled those on T. Muir; but there was no proteolysis of gelatine, and drug-resistance again included 30 μg. streptomycin, 100 μg. P.A.S., 5 μg. I.N.H., cycloserine and ethionamide as well as penicillin and sulphonamide; but erythromycin and soframycin, and, to a lesser extent, chloramphenicol and oxytetracycline were effective.

The organism was classified as a photochromogen, Runyon's Group I.

GEORGE BROWN AND ROBERT FAULDS

These two strains were noted among sputum cultures because of their intense resistance to I.N.H. exceeding 50 μg.; though they were sensitive to streptomycin, P.A.S., and viomycin. The growth was slow at 37°C, taking 2 - 3 weeks, and while Brown's culture was orange in the dark, that of Faulds was uncoloured even in the light. Both were catalase -, and both grew on agar as well as Lowenstein-Jensen. The bacilli were acid- and alcohol-fast but very pleomorphic. They were classified respectively as a scotochromogen of Group II in the case of Brown, and a Battey strain of Group III in the case of Faulds.

Runyon has pointed out, however, that nobody has ever isolated chromogenic Mycobacteria from actual cultures of tubercle bacilli as has been done with the merely I.N.H.-resistant strains, and that remarkably distinctive as the latter are from the parent tubercle bacilli, the distinction is trifling compared with the utterly different pigmented and pleomorphic Anonymous Mycobacteria.

Wolinsky [474] comments that these organisms were noticed occasionally even before the advent of tuberculostatic drugs, and he suggests that the increasingly frequent discovery now is due to three factors:-

1. Ordinary tuberculosis is decreasing.
2. Increased interest leads to their recognition.
3. Lengthy cultural procedures for drug tests for tubercle bacilli fortuitously favour the recognition of these rarer Mycobacteria too.

If the chromogens were in any way constant in treated tuberculosis cases the Slope Diffusion Test should contribute to their recognition by providing gradients of drugs with the tubercle bacilli inhibited; but we have found no pigmented colonies in the inhibitory zones even when cultures have been left for weeks at room temperature.

Nevertheless there is a curious tendency commented on by others [380] for these abnormal Mycobacteria to occur sporadically among routine Lowenstein-Jensen cultures, and especially in crops during certain months of the year, and their role in disease, let alone their relationship to true tuberculosis, remains an enigma.

NATURALLY-RESISTANT STRAINS OF TUBERCLE BACILLI

Mycobacterium bovis has been recognised for some years to be relatively more resistant to P.A.S. than the human type. Natural isoniazid-resistant strains of tubercle bacilli also appear to be not uncommon in Africa and India, and sometimes this is associated with avirulence.

Natural Streptomycin-resistance in Myco. tuberculosis

Figure 39 showed a single large colony of Myco. tuberculosis growing luxuriantly on Lowenstein-Jensen medium with 100 µg. of streptomycin at its base. If we accept that drug-resistance arises from the multiplication of these pre-existing mutants it is not unreasonable to assume that occasionally a strain will be found in which they are already in the majority from the first encounter. Such a circumstance will confer natural drug-resistance and cases have been described for streptomycin also; but unlike isoniazid they are extremely rare. The following example was described by the author in 1950:

Natural Streptomycin-resistant Tuberculosis in a Newborn Child[437]

This baby, who was born prematurely and breast-fed by an untreated tuberculous mother, developed miliary tuberculosis of the lungs, and an enlarged liver and spleen, when only eight weeks old. Daily injections of Streptomycin for three months, totalling 11½ Gm., were unavailing, and death occurred after increasing pulmonary consolidation.

Gastric washings were obtained at the outset and just before death, and Myco. tuberculosis was cultured from both. The first specimen showed no apparent inhibition by 6 μg/ml. of Streptomycin in Dubos medium, the highest concentration used. As the control H37Rv was sensitive to ½ μg/ml. the relative degree of resistance was not less than twelve times that of the control culture. It was realised that a sensitivity test with a wider range than usual would be necessary, and the Slope Diffusion Test was therefore used in addition to study the resistant mutants which seemed likely to be present.

The results of the Dubos and Slope Diffusion Test on the cultures from the baby before and after treatment, compared with the control culture H37Rv were as follows:-

TABLE 40

STREPTOMYCIN SENSITIVITY TESTS ON MYCO. TUBERCULOSIS

Strain	Dubos Method (μg/ml.)	Slope Diffusion Method: Inhibitory Zones at 4 weeks (cm.)	Slope Diffusion Method: Resistant Mutant Colonies at 6 weeks
H37Rv	$\frac{1}{5}$	2.75	0
Baby O'D before treatment	>6	1.5	55
Baby O'D after treatment	>6	Nil	Uncountable

It will be seen that even though the initial Streptomycin-resistance was high, it was much enhanced by three months' Streptomycin therapy just as occurs in the vast majority of cases which have initial sensitivity. The final intense resistance is shown in the photograph, where the baby's strain obtained prior to death is compared with H37Rv, both streaked on a Lowenstein-Jensen plate at right angles to a gutter containing Streptomycin 100 μg/ml. solution, the sealed plate being incubated at 37°C for two months (Fig: 50).

In May 1948 when this infant was infected, Streptomycin had not become available for general hospital use in Britain, the supply being restricted to a few research units. It was therefore most unlikely that infection had come from anyone harbouring tubercle bacilli rendered resistant by therapy. Post-mortem was refused, and the mother, who was the probable source of the infection, unfortunately refused treatment and left for Eire before any of her sputum could be obtained for culture.

As long ago as 1947 [352] and 1948 [497] it was shown that the susceptibility to streptomycin was by no means uniform throughout a strain of tubercle bacilli, and the Slope Diffusion Test indicates clearly that the initial resistance of this case as shown by the Dubos method is not attributable to a homogeneous culture but really represents a very high proportion of resistant

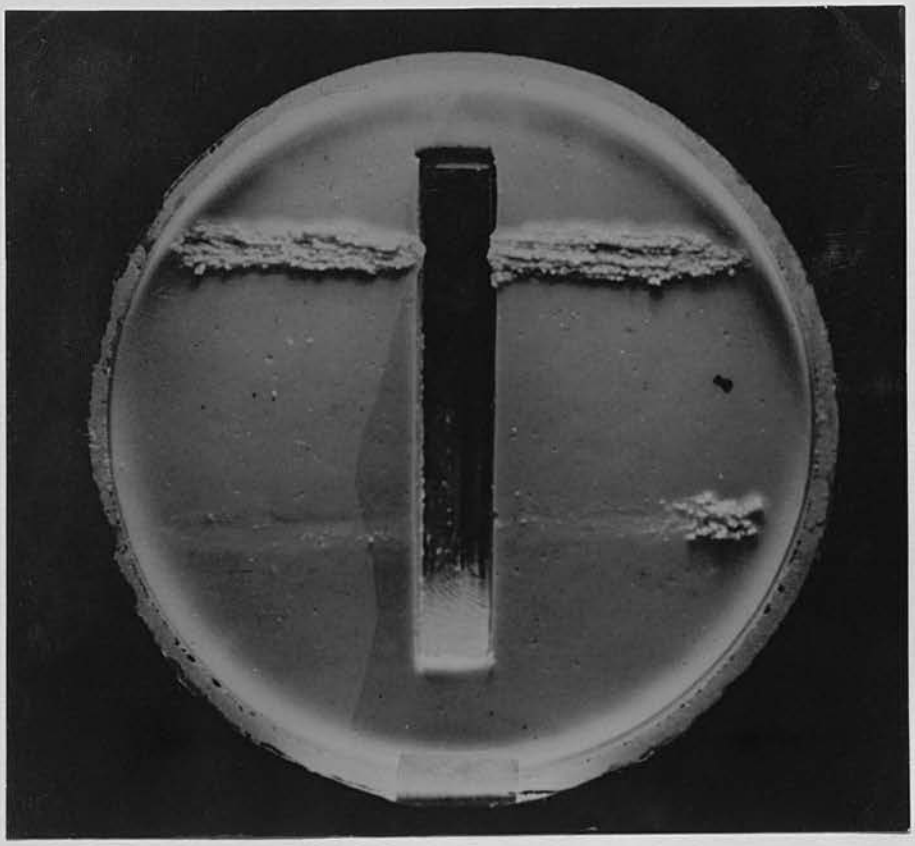


Fig: 50. *Comparison of the resistant strain from Baby O'Donnell with the sensitive strain H37 Rv of Myco. tuberculosis on a Lowenstein-Jensen plate with a gutter containing 100 µg./ml. of streptomycin.*

The growth of the control culture (H37 Rv) has been restricted to one edge of the plate, while the resistant specimen has grown uninhibited up to the margins of the gutter on both sides.

mutants, which by overgrowth come to dominate the final result. On the solid slopes this takes between four and six weeks and can be observed; but in the liquid culture of Dubos with its more rapid and all-or-nothing growth such distinctions are not detectable.

The full extent of the potentialities for drug-resistance of the tubercle bacilli from this baby was shown by a subculture of the confluent growth on the above gutter-plate, which was found to be resistant in turn to no less than 1,000 μg./ml. on a repetition of the test with a stronger streptomycin solution. This and the previous experiments suggested strongly that the progressive development of resistance in Myco. tuberculosis depends on mutants, just as was seen with strains of Esch. coli in Chapter 8. It seemed possible that the actual numbers of resistant and sensitive tubercle bacilli in a strain could be estimated, therefore, by an analogous technique using plate counts.

EXPERIMENT 36 Plate Counting of Resistant Colonies of Myco tuberculosis.

1. Estimate of the number of resistant bacilli in Strain H37Rv.

A one-month old Dubos culture of the control strain H 37Rv (Opacity Brown No: 2 tube) was homogenised by 3 hours shaking at 6-10 beats per second with two $\frac{1}{8}$" glass beads. A standard 4 mm. loopful of a 1/50 dilution was spread widely on 8 sets of Lowenstein-Jensen plates containing 1, 10, and 100 μg./ml. streptomycin respectively, and a similar standard 4 mm. loopful of a 1 in 100,000 dilution was spread on 2 plain Lowenstein-Jensen plates to give total counts of the inoculum. All were incubated at 37°C for 6 weeks.

Inoculum Counts:-

16 38 Average 27

Number of bacilli per loopful of original culture =

Average count X Dilution =

2,700,000

Resistant Colony Counts:-

Concn. of Streptomycin	Counts								Average
100 μg./ml	0	0	0	0	0	0	0	0	0
10 μg./ml	0	0	0	0	0	0	1	0	0.125
1 μg./ml.	94	202	120	210	190	150	250	154	171.25

Dilution = 50 in each case.

Therefore in an inoculum of 2,700,000 bacilli there are:-

Resistant to 100 μg.	nil
" " 10 μg.	6.25
" " 1 μg.	8,562.5

Which gives a very approximate rate for resistant tubercle bacilli in strain H37Rv of:-

Resistant to 100 μg.	nil in $2\frac{3}{4}$ million
" " 10 μg.	about 1 in 400,000
" " 1 μg.	about 1 in 3,000

2. Estimate of the number of resistant bacilli in patient's strains

A count of the proportion of resistant colonies of Myco. tuberculosis in the strains from the baby before and after treatment was made in a similar manner, suspensions from the solid cultures being attempted by prolonged shaking in Dubos medium as in the previous experiment. Owing to the tendency of tubercle bacilli to re-adhere such a count can only be a conjecture; but estimates of the resistant organisms in the two specimens were made by spreading standard 4 mm. loopfuls of a 1 in 20,000 dilution on 10-cm plates containing 40 ml. Lowenstein-Jensen medium with 0, 10, and 100 μg./ml. streptomycin respectively. Multiplying by the dilution factor was therefore unnecessary as only approximate direct ratios were being attempted, and estimates of the resistant bacilli in the two specimens were as follows:-

Initial Specimen

Resistant to	10 μg./ml.	2.6%
" "	100 μg./ml.	0 %

Final Specimen

" "	10 μg./ml.	3.3%
" "	100 μg./ml.	1.6%

which compares with the very rough estimates of 1 in nearly $\frac{1}{2}$ million resistant to 10 μg./ml., and 1 in nearly $2\frac{3}{4}$ million resistant to 100 μg./ml. in the typical "sensitive" strain H37Rv.

It must be emphasised, however, that the strong mutual adhesiveness of Mycobacteria probably makes supposed counts of bacilli based on the number of colonies quite misleading except for broad comparisons of gross resistance and sensitivity as in the example above. However small, symmetrical and discrete, the colonies are much less likely than Esch. coli to be from a single organism. There seems little to be gained by plate counts, therefore, over the generalisations possible from counting resistant mutants as seen in the Slope Diffusion Test.

EMERGENT RESISTANT MUTANTS

Apart from the relatively rare naturally occurring resistant Mycobacteria which we have described, one must presume that all drug-fast strains originated during treatment from surviving mutants. The discovery that penicillin-resistant staphylococci come from mutants suggested, of course, that a similar accommodation could occur in any bacterial species exposed to widespread use of a chemotherapeutic drug. With tubercle bacilli resistant to streptomycin, isoniazid or P.A.S. emerging among cases under treatment as described in the last Chapter, it is inevitable that they are now being reported throughout the world among tuberculous cases when first diagnosed. Token use of isoniazid in India and Nigeria for both prophylaxis and large-scale treatment has, for instance, provided a huge pool of live tubercle bacilli which have "experienced the drug". It would seem that the eventual control of the disease may have been sacrificed to immediate expediency.

The advantages of solid media for sensitivity tests on tubercle bacilli were described in the last Chapter. Mitchison and others [319], however, showed that the actual 'minimum inhibitory concentration' or 'M.I.C.' of a strain could vary between laboratories from such factors as differing preparation and storage of media, and though their work was

with sensitive strains of Mycobacterium bovis such variations are inevitable in any supposedly quantitative biological test such as Mycobacterial sensitivity tests are. Cruickshank and Stewart [83] therefore suggested that resistance ratios would be a more reliable index than absolute values. Strains are cultured on eight slopes incorporating doubling dilutions of a drug, the control strain H37Rv., being treated exactly alike, and the minimum inhibitory concentration is expressed as a fraction or multiple of that of the control strain.

There are, however, contrary arguments for absolute values as the criteria of resistance when performing serial tests on the same patients' strains very frequently and even monthly. The specimens by their numbers act as "internal controls" as with Wassermann Reactions read similarly in bulk, in fact minor inconsistancies in the control H37Rv., itself are eliminated, and that the results are sufficiently reproducible is shown by a comparison of 295 such tests with resistance ratios which shows 85% correlation (Table 41).

For our special purpose of studying the development of resistance through mutants we have used a combination of the Slope Diffusion Test and incorporated media throughout a decade. The Slope Diffusion Test gives the overall sensitivity of the bacterial population to an antibiotic in a roughly quantitative way, which is, however, infinitely

TABLE 41

DRUG-RESISTANT TUBERCULOSIS

IN EASTERN GLASGOW, 1963.

	Assessed by Slope Diffusion Test	Additionally by Resistance Ratios Test	Total
Sensitive	153	-	153
Resistant to:-			
Streptomycin	15	2	17
P.A.S.	3	1	4
I.N.H.	7	2	9
Viomycin	2	-	2
Cycloserine	-	-	-
Ethionamide	-	-	-
Strept. & I.N.H.	23	12	35
Strept. & P.A.S.	6	2	8
P.A.S. & I.N.H.	4	2	6
Strept. & Vio.	1	-	1
Strept., P.A.S., I.N.H.	21	9	30
Strept., I.N.H., Ethionamide	2	-	2
Strept., I.N.H., Cycloserine	1	-	1
Strept., P.A.S., I.N.H., Vio.	4	2	6
Strept., P.A.S., I.N.H., Cyclo.	5	2	7
Strept., P.A.S., I.N.H., Ethion.	2	2	4
Strept., P.A.S., I.N.H., Cyclo., Vio.	2	-	2
Strept., P.A.S., I.N.H., Cyclo.. Ethion	1	-	1
Strept., P.A.S., I.N.H., Vio., Ethion	5	1	6
Strept., P.A.S., I.N.H., Vio., Cyclo., Ethion	1	-	1
	105	37	
	258	37	295

graduated, and this has a particular application when matching strains in those suspected of cross-infection. Its use will be seen in the family outbreaks described later in this Chapter, and especially strikingly in the almost certain occupational cross-infection seen in the case of Rinaldi and Baume.

It was shown in earlier Chapters that strains of bacteria were heterogeneous, and in Chapter 10 the point was made, as by others [127, 211, 317, 352, 411, 439], that a mixture of sensitive and resistant tubercle bacilli will appear at least partially resistant by ordinary tests [317, 318]. We have followed the emergence of resistant mutants by two methods. One is by counting the colonies on media incorporating the same concentrations of a drug, streptomycin, P.A.S., or isoniazid, month after month. 3 μg./ml., streptomycin, 2 μg./ml., P.A.S., and 1 μg./ml., isoniazid have been found empirically to be the usual thresholds for isolating significantly resistant mutants. For streptomycin and isoniazid the amounts are related to attainable blood levels of the drugs; but for P.A.S., they are a compromise with reports that even low resistance is clinically significant [422].

The other method, which we are inclined to think is more perceptive, at least for streptomycin-resistant mutants, is to read the Slope Diffusion Test's main growth at 4 weeks'

incubation, and then re-examine at 6 weeks for the presence of resistant colonies in the inhibitory zone. The results are read as the height of the zone in inches plus the mutant colony count thus:- $1\frac{1}{4}$" + 6. Table 42 shows 12 strains investigated by this method and also by that of the Resistance Ratios in parallel. It will be seen how a relatively few mutants can drastically influence the Ratios:-

TABLE 42

SIGNIFICANCE OF RESISTANT MUTANTS IN TWELVE DRUG-SENSITIVITY TESTS WITH STREPTOMYCIN

Case	Date	Resistance Ratio	Slope Diffusion
Jn. Anderson	24/1/64	16X = Res.	$\frac{1}{8}$" + 3 = Sens $\pm$
Robt. Fisher	28/1/64	2X = Sens.	1" + 1 = Sens $\pm$
Patrick Flynn	8/11/63	1X = Sens.	$\frac{1}{4}$" + 0 = Sens $\pm$
Robt. Frankland	27/8/63	(a) 4X = ? Res. (b) 8X = Res.	$\frac{3}{4}$" + 6 = Sens $\pm$
Robt. Gibson	27/11/63	16X = Res.	$\frac{3}{4}$" + 8 = Sens $\pm$
Henry Halbert	20/3/63	(a) 4X = ? Res. (b) 2X = Sens	$\frac{1}{8}$" + 5 = Sens $\pm$
Alfred Hughes	4/6/63	(a) 4X = ? Res. (b) 4X = Res.	$\frac{1}{8}$" + 2 = Sens $\pm$
Jn. Lynch	9/1/64	16X = Res.	$\frac{1}{8}$" + 4 = Sens $\pm$
Jas. McGowan	6/11/63	8X = Res.	$\frac{3}{4}$" + 10 = Sens $\pm$
Pat McLusky	12/3/63	16X = Res.	$\frac{3}{4}$" + 14 = Sens $\pm$
Andrew Wales	12/10/63	8X = Res.	$\frac{1}{8}$" + 6 = Sens $\pm$
George Park	10/9/63	(a) 4X = ? Res. (b) 1X = Sens	$\frac{1}{8}$" + 0 = Sens $\pm$

Abbreviations: Res = resistant, ? Res. = doubtfully resistant (in Resistance Ratios), Sens = sensitive, Sens $\pm$ and Sens $\pm$ = diminishing, and greatly diminishing sensitivity, respectively (by Slope Diffusion).

TABLE 43

TWENTY-FIVE EXAMPLES OF THE SIGNIFICANCE OF MUTANTS IN SERIAL STREPTOMYCIN-SENSITIVITY TESTS

A Comparison of Resistance Ratio and Slope Diffusion Methods to show the future significance of the Mutant Counts in the latter as read at six weeks.

Name		July/Dec. '62	Jan./June '63	July/Dec. '63	Jan./June '64
1. Walter Brown		13/6	12/4	27/9	25/2
	Ratios:	-	4X = Res.	8X = Res.	16X = Res.
	S.D:	1" + 4m = Sens ±	1" + 1 = Sens +-	¾" + 3 = Sens ±	¼" + 1 = Sens ±
2. George Baird		1/11		6/7	24/3
	Ratios:	16X = Res.		8X = Res.	16X = Res.
	S.D:	¼" + 10= Res.		0" = Res.	0" = Res.
3. Henry Caddies		1/11		12/7	17/3
	Ratios:	2X = Sens		16X = Res.	16X = Res.
	S.D.:	½" + 1 = Sens ±		¼" + 6 = Res.	0" = Res.
4. Wm. Clark		1/11	31/5	31/12	
	Ratios:	1X = Sens	1X = Sens	2X = Sens	
	S.D:	1" + 0 = Sens	¾" + 0 = Sens	¾" + 0= Sens	
5. Denis Docherty		29/9	19/1	20/11	3/3
	Ratios:	-	(a)32X = Res (b)16X = Res	16X = Res	16X = Res
	S.D.:	0"= Res.	0" = Res	¼" + 4= Res	0" = Res

TABLE 43 (Contd.)

Name		July/Dec, '62	Jan./June '63	July/Dec. '63	Jan./June '64
6. George Docherty		6/9		20/9	
	Ratios:	1X = Sens		16X = Res	
	S.D:	$\frac{3}{4}$" + 3= Sens ±		$\frac{1}{4}$" + 8= Res	
7. George Fitzpatrick			12/3	24/9	21/1
	Ratios:		32X = Res.	16X = Res.	16X = Res.
	S.D:		$\frac{1}{4}$" + 1 = Sens ±	$\frac{1}{2}$" + 3= Sens ±	$\frac{1}{2}$" + 5= Sens ±
8. Gerald Harvey		20/12	24/6		15/4
	Ratios:	2X = Sens	(a) 4X = ? Res		4X = ? Res
			(b)16X = Res		
	S.D:	$\frac{3}{4}$" + 0 = Sens	$\frac{1}{2}$" + 0 = Sens ±		0" = Res
9. James Henderson			11/4	29/11	
	Ratios:		1X = Sens	1X = Sens	
	S.D.		$\frac{3}{4}$" + 0 = Sens	$\frac{3}{4}$" + 0 = Sens	
10. James Kelly			12/3	25/11	30/1
	Ratios:		16X = Res	16X = Res	16X = Res
	S.D:		0" = Res	0" = Res	0" = Res
11. Sarah Kelly		2/8	10/4	31/7	11/2
	Ratios:	32X = Res	16X = Res	16X = Res	16X = Res
	S.D:	$\frac{1}{2}$" + 0 = Sens ±	$\frac{1}{4}$" + 0 = Res	$\frac{1}{4}$" + 2= Res	$\frac{3}{4}$" + 2= Sens ±
12. Patrick McCarron		22/11		1/11	3/3
	Ratios:	16X = Res		8X = Res	16X = Res
	S.D:	2" + 0 = Sens		$\frac{3}{4}$" + 3= Sens ±	$\frac{3}{4}$" + 1= Sens ±

TABLE 43 (Contd.)

Name		July/Dec. '62	Jan./June '63	July/Dec. '63	Jan./June '64
13. Janet McCormack		23/10	4/6	12/11	5/3
	Ratios:	32X = Res	32X = Res	16X = Res	16X = Res
	S.D:	0" = Res	0" = Res	0" = Res	0" = Res
14. Hector McDonald			20/4	24/9	
	Ratios:		16X = Res	8X = Res	
	S.D:		$\frac{1}{2}$" + 0 = Sens ±	$\frac{1}{4}$" + 0= Res	
15. James McGhee		25/9	13/6	18/12	
	Ratios:	32X = Res	16X = Res	16X = Res	
	S.D:	0" = Res	$\frac{1}{2}$" + 0 = Sens ±	$\frac{3}{4}$" + 12= Sens ±	
16. Thomas McIntosh			3/4	13/11	
	Ratios:		16X = Res	16X = Res	
	S.D:		0" = Res	$\frac{3}{4}$" +10= Sens ±	
17. James McNamara		6/9			22/1
	Ratios:	2X = Sens			1X = Sens
	S.D:	$1\frac{1}{4}$" + 0= Sens			1"+ 0 = Sens
18. James Millan			22/4	22/10	
	Ratios:		16X = Res	8X = Res	
	S.D:		$\frac{3}{4}$" + 0= Sens	$\frac{1}{2}$" + 3= Sens ±	
19. Mary Murray			30/1	4/7	
	Ratios:		1X = Sens	(a) 4X = ? Res (b) 16X= Res	
	S.D:		$\frac{3}{4}$" + 4 = Sens ±	$\frac{1}{2}$" + 0= Sens ±	

Table 43 (Contd.)

Name		July/Dec. '62	Jan./June '63	July/Dec. '63	Jan./June '64
20. Cornelius Petrie		8/8	18/6		18/3
	Ratios:	1X = Sens	(a) 8X = Res (b) 16X = Res		16X = Res
	S.D:	1" + 3= Sens ±	0" = Res ¾" + 3 = Sens ±		0" = Res
21. Malcolm Stewart		14/9		13/11	8/2
	Ratios:	16X = Res		16X = Res	16X = Res
	S.D:	¾" + 10= Sens ±		¾" + 4= Sens ±	¼" + 5= Res
22. Margaret Taylor		6/7	8/2	3/10	
	Ratios:	1X = Sens	1X = Sens	2X = Sens	
	S.D:	1" + 1= Sens ±	1" + 12 = Sens ±	¾" + 4= Sens ±	
23. James Travers			21/5	6/12	
	Ratios:		8X = Res	8X = Res	
	S.D:		¾" + 0 = Sens	¼" + 0 = Res	
24. David McDougall		14/12	10/1	19/6	
	Ratios:	32X = Res	16X = Res	16X = Res	
	S.D:	¼" + 4= Res	0" = Res	0" = Res	
25. Alice Withers			19/1	24/8	28/2
	Ratios:		16X = Res	16X = Res	
	S.D:		¼" + 4 = Res	0" = Res	0" = Res
(Compare husband Eric Withers *			16/3		19/5
	Ratios:		1X = Sens		No
	S.D:		¾" + 0 = Sens		growth

TABLE 43 (Contd.)

Abbreviations: *Day and month of test are given thus:- "6/7"*
The sensitivity tests were performed by one or both methods, as far as possible at six-monthly intervals.

"Ratios" = Resistance Ratio Tests [83].

"S.D" = Slope Diffusion Tests as described in Chapter 10, the reading of the zone of inhibition being given thus in inches:- " $\frac{3}{4}$" followed by a count of the resistant colonies within that zone read up to six weeks thus: - ' + 4'.

Results are given thus:-

	'Sens'	*= Sensitive*
	'Res'	*= Resistant*
	'? Res'	*= Doubtfully resistant (by Ratios Method)*
	'Sens $\pm$	*= Diminishing sensitivity*
and	' $\overset{+}{-}$ '	*= Greatly diminishing sensitivity (by Slope Diffusion)*

* Mr. and Mrs. Withers had probably been simultaneously infected ten years previously when staying with her brother, Mr. Shearer, who had died from tuberculosis. There is no record of any tests on his bacilli. In 1963 Eric Withers had relapsed after many years' quiescence, which he now appears to have regained. His wife has been more or less continuously ill. The above test is interesting as implying that his relapse was not a superinfection from his wife, as it could have been if the bacilli had been as resistant as in her more recent specimens.

COMMENTARY ON TABLE 43

This table shows the advantages of the Slope Diffusion Test read at four weeks and again at six weeks, when particular note is made of the emerging mutants. By using both tests, Resistance Ratios and Slope Diffusion, and carrying out a series at roughly six-monthly intervals, the consistency of the results in the Slope Diffusion Test can be seen.

Wholly or Partially Sensitive Strains

It will be seen that Cases No: 4, 9, and 17 were consistently sensitive by both the Resistance Ratio and Slope Diffusion methods and that the latter test also revealed no mutants to mar this interpretation. In No: 22, however, while both tests are satisfactorily sensitive on three successive occasions, the Slope Diffusion with its additional reading at six weeks shows an ominously progressive rise in mutants. In Nos: 7 and 12 their presence probably explains the discrepancy between the two tests, Slope Diffusion breaking down the "resistance" of the Ratios into an overall sensitive growth with a few mutants arising month by month, and in No: 19 the abrupt transition from sensitive to resistant in the Ratios is foretold in the Slope Diffusion by the presence of mutants and a dwindling zone of sensitivity.

Resistant Strains

Similarly with the resistant cases for, while Nos: 5, 10, and 13 are overwhelmingly resistant by both methods, in Nos: 2, 14, 18, 24, and 25 the Slope Diffusion Test shows that the onset is more gradual than the Ratios alone would suggest, being preceded by a diminishing zone of inhibition and often a rising mutant count as well. In Nos: 15 and 16 such analysis of the Slope Diffusion Test suggests that resistance is in fact more borderline than the broad picture of the ratios would suggest.

Strains in Transition

The more or less gradual transformation from sensitivity to resistance is seen in Nos: 3, 6, and 8 by both Resistance Ratios and Slope Diffusion methods; but the Slope Diffusion discloses the same phenomenon in Nos: 1, 21, and 23, and in Nos: 1, and especially 21, the rise in mutants is seen preceding diminishing sensitivity. In Nos: 11 and 20 this critical discrimination of the Slope Diffusion Test shows by alternating results that resistance is by no means finally established.

By recognising and reporting in this way the significance of these resistant mutants on both our Slope Diffusion Tests and also on media incorporating the drugs,

we have gone some way to meet Canetti's objections to the usual tests. His ingenious "Proportion Method" [60] involves making successive dilutions of the inoculum of tubercle bacilli to obtain counts of the resistant colonies at three weeks, so that, for instance, 1% of the population resisting 4 μg. streptomycin, 0.5 μg. P.A.S., or 0.2 μg. isoniazid, or proportionately higher counts at lower concentrations, would indicate clinical resistance to these drugs. Such an elegant test is as precise as any yet devised; but it is unfortunately hardly practicable for a busy hospital laboratory, in particular as regards the safe preparation of the inoculum dilutions.

It has been claimed that to report sensitive organisms at all among resistant ones may mislead as the benefits of the drug will not be lasting; but where other drugs are already less effective, even transient benefits are justified if only as a prelude to surgery. That the combination of the Slope Diffusion Tests, with Mutant Counts included, gives a realistic assessment is shown by the serial results for the long-term cases given in Table 43 . The clinical correlation is demonstrated in more detail in the following example, aptly termed a "salvage case", in which a combination of Slope Diffusion Tests and colony counts on drug-incorporated media have been employed throughout.

EMERGENCE OF DRUG-RESISTANT MYCO. TUBERCULOSIS IN AN INDIVIDUAL

Tuberculosis is unique among infectious diseases in that its chronic nature and intractibility under treatment lend themselves to providing a perfect site for the parasite's evolution in a single host over many years. In such an ecological niche repeated mutations can occur, and, in an extreme example such as the following case five years may suffice to breed bacilli resistant in more or less degree to all the ten currently available drugs.

A Case of Extreme Multiple Drug-resistance

Miss Helen Chisholm was a factory machinist whose sister had died of pulmonary tuberculosis in 1947. Two years later, when she was 23, a haemoptysis showed that she too had the disease in the right upper lobe but two months in hospital appeared to give quiescence.

A year and a half later radiography showed activity again and spread to the left upper lobe. Two months were again spent in hospital with collapse therapy, and then 18 months at home on streptomycin and P.A.S. 41 Gm. of streptomycin were injected, aimed at controlling the left lung infection before surgery on the right side. But now, in May 1953, another haemoptysis showed cavitation on the left side, and isoniazid and P.A.S. replaced the streptomycin

and P.A.S. Four months later the sputum was negative, and quiescence was again assumed. Next year the cavitation had increased, streptomycin and P.A.S. were again introduced and a total of 50 Gm. streptomycin were given at home during 1954.

In 1956 Miss Chisholm married Mr. Anderson, who died the next year of pneumonia after influenza, leaving her a daughter. The subsequent winters of 1957, 1958 and 1959 were all spent in hospital with detiorating health. During these years there were in vitro resistance of the tubercle bacilli to streptomycin and isoniazid, and also increasingly though to a lesser extent to P.A.S., and the accompanying table shows how the lowering of sensitivity to all three drugs is due to the accumulation in each case of resistant mutants.

By 1959 cycloserine had replaced the earlier drugs as the accompaniment to P.A.S., and in June 1959 viomycin was added too and gave some improvement with rest at home. In August the P.A.S. was stopped because of nausea; but was hastily resumed in November when two large cavities appeared at the right apex. It will be seen in the table how the Slope Diffusion Test with cycloserine reveals that this drug too had lost its effect, the tubercle bacilli being only moderately sensitive to it and to P.A.S., and quite resistant to streptomycin and isoniazid (11.11.1959). The untimely three months' stoppage of P.A.S. could have contributed to

this emergence of cycloserine-resistance; but fortunately viomycin retained its effect. The patient felt well during 1960; but the sensitivities of the bacilli remained the same, and the cavities growing steadily bigger spread by September also to the right side.

During 1961 resistance to viomycin began to appear and was explicable by the increasing counts of resistant mutants shown in the table, though this was offset to some extent by a transient return of sensitivity to P.A.S. in March, and to cycloserine in May and June, presumably due to over-riding back-mutations. By June 1962 the patient had been alternating between bed at home and in hospital with a variety of drugs such as ethionamide and pyrazinamide, and the tubercle bacilli were now, after 14 years, resistant to no less than 8 drugs:- streptomycin, isonaizid, pyrazinamide, viomycin, cycloserine, terramycin, ethionamide, and kanamycin. P.A.S. and Dipasic, which still showed some in vitro effect, were then used, and in the next month, July, a new antibiotic Fucidin providentially arrived from Denmark, where early in vitro tests had suggested some tuberculostatic action. 1½ Gm. were given daily, and by October the bacilli were not only still sensitive to fucidin, but showed reversion of sensitivity to cycloserine and partially to viomycin and kanamycin. The list of ineffectual drugs, however, now included P.A.S. and Dipasic, the two drugs used with the fucidin.

TABLE 44

SIX YEARS' DRUG-SENSITIVITY TESTS ON TUBERCLE BACILLI FROM HELEN ANDERSON

(OVERALL SENSITIVITY BY SLOPE DIFFUSION; MUTANT COUNTS ON DRUG-CONTAINING MEDIA).

Date	Streptomycin	mutants 30	mutants 100	P.A.S.	mutants 2	mutants 10	mutants 100	I.N.H.	mutants 1	mutants 5	mutants 10	mutants 50	Viomycin	mutants 100 μg.
'56 Oct.	R 0"	∞		S 2"				R 0"	∞				S ¾"	
'57 Aug.	-	-		-				-	-				-	
'58 May	-	-		-	8			-	-				-	
July	-	-		S ± 1"	∞	3		-	-	∞			-	
Sept.	-	-		-		12		-	-	-	∞		S ± ½"	
Oct.	-	-	12	-		11		-	-	-			-	2
'59 Sept.	-	-	20	-		1		-	-	-			S ¾"	
Nov.	-	-	50	-		50	20	-	-	-			-	
'60 Mar.	-	-	4	-		10	5	-	-	-			-	
Nov.	S ± ¼"	12		-	30	3		-	-	-			-	6
Dec.	-	30	3	-	20	10		-	-	-			-	6
'61 Jan.	-			-	50	30		-	-	-			S ± ½"	
Feb.	-	5	3	-		1		-	-	-			-	
Mar.	-	15		-	10			-	-				R 0"	30
Apr.	-	8	3	-		0		-	-		50		-	50
May	R 0"	∞	30	-	40	6		-	-	∞				
June	-	-	30	-		4	5	-	-		∞		-	100
Aug.	-	-		-			3	-			50			
Sept.	-	100		-		36	0	-		∞	∞		-	100
Oct.	-	30		-			1	-			100			
Nov.	-	50		-		12	5	-					-	25
Dec.	-	∞		-		8	0	-		∞			-	12
'62 Jan.				-		4	0						S 1"	
Feb.	-	∞		-		20	2	-		∞			-	3
Mar.				S ± ½"		∞	50						S ± ½"	6
Apr.	-	∞		S ± ¾"		7	1	-	-	∞			-	
Nov.	-			-		20	12	-	-	∞	10		-	30

(*Resistance Ratios at this date 2nd. Nov. 1962:-*

Streptomycin 32X, P.A.S. 32X, I.N.H. 800X, Viomycin 4X, Ethionamide 16X, as resistant as control culture H37 Rv. Cycloserine 1X, i.e. sensitive).

R = resistant, S = sensitive, S ± = moderately and S ± = only slightly sensitive respectively. - = result as before.

Summary

Mrs. Anderson's story has been given in detail because her infection coincided with the discovery of the tuberculostatic drugs, and her subsequent fifteen years' history is that of the chemotherapy of tuberculosis.

Drug-sensitivity tests on the tubercle bacilli were not done for seven years by which time streptomycin- and isoniazid-resistance was already present. The table shows the sequence of events in the eight years since, and demonstrates how by using the quantitative Slope Diffusion Test and by counting resistant mutant colonies, the mutational origen of the drug-resistance can be unequivocally proved.

Isoniazid-resistance has continued irreversibly in the whole eight years since 1956, and streptomycin-resistance has been similar except for temporary remissions early in 1961 - evidence that back-mutations, though infrequent, do occur with streptomycin in contrast to isoniazid. P.A.S.-sensitivity has declined steadily since 1958, and a similar story of rising resistance has made the population of tubercle bacilli also immune at various times to viomycin, cycloserine, Dipasic, and ethionamide.

The Rise of Drug-resistant Tuberculosis

EMERGENCE OF RESISTANT TUBERCLE BACILLI IN THE COMMUNITY

Tuberculosis has always been relatively common in Scotland, and there was a two-thirds increase during the last war. After the war the fall in incidence lagged far behind that of the rest of Western Europe.

That there was still a big reservoir of undetected tuberculous infection in Glasgow could hardly be doubted, just how big could only be shown by the Mass Radiographic Survey which took place in 1957 [161]. In five weeks 714,915 people were examined, and those with X-ray evidence of disease were just over 1%, one third of these being active cases. But these 2,565 active cases were by no means evenly distributed. A rate of 0.15% for the University contrasted with 2% for the prison, 3% for a mental hospital, and no less than 10% among the 7,000 dwellers in Lodging Houses. In 1962 twenty new cases were still being found in Glasgow every week, and typically half of these had missed the Mass Survey, and more than half came from the lodging houses. 40% had positive sputa, and one in eight bilateral cavities.

In the same year as the Mass Survey drug-resistant tuberculosis was already giving cause for concern. Bruce,

Cuthbert and Ritchie [44] had found 125 cases, 59 of them resistant to more than one drug. Twenty were untreated cases, and five had been in contact with a treated case. Tinne [434] had found among 106 newly diagnosed cases in the city 9 resistant to streptomycin, and 1 each to P.A.S. and I.N.H., while among 263 cases already being treated more than half harboured resistant strains, in one case resistance being to no less than four drugs (Table 39).

Reports of primary drug-resistance in tuberculosis for 1957 and the two following years were very similar for other cities as far apart as Madras (8%), Strasbourg (11%), and Athens (11%), and at an international conference in 1961 [213] nine countries agreed that their primary resistance rates of 3 to 11% had become general.

RESISTANT CROSS-INFECTIONS IN FAMILIES

The insidious onset of tuberculosis may make the source extremely difficult to trace; but where several cases occur in one family there was always the presumption that one had infected another. This now receives strong support when the pictures of drug-resistance in the strains exactly match or could formerly have matched, having regard to the very long incubation period of the disease, on which, incidentally, this sheds new light. The following four family outbreaks exemplify this, in one case the family dying out with the resistant infection.

FAMILY 1. MRS. ODGER AND HER TWIN DAUGHTERS
(Fig: 51 and Table 45)

Mrs. A. Odger, the mother of three daughters, was infected with tuberculosis in the left lung in 1944. This responded to a thoracoplasty in 1945 with subsequent P.A.S. and I.N.H., but by May 1954 the infection had spread to the right lung with cavitation.

Streptomycin, P.A.S. and I.N.H. for two years, and then Streptomycin and P.A.S. or dipasic or both during 1957 again produced quiescence; but in June 1958 the bacilli were found resistant to Streptomycin and I.N.H. Streptomycin P.A.S. and dipasic continued to be used, however, until August 1960 when a report of the culture's resistance to Streptomycin, I.N.H. and dipasic forced a substitution of cycloserine and ethionamide, with six months in hospital.

In the winter of 1961 unchanged resistance to the three primary drugs induced the use of viomycin, cycloserine and dipasic, and in June 1962 ethionamide had to replace cycloserine which in turn had become useless. In Feb. 1963 resistance to Streptomycin, I.N.H., P.A.S. and dipasic, and intolerance to cycloserine and ethionamide have dictated kanamycin and pyrazinamide as the current treatment.

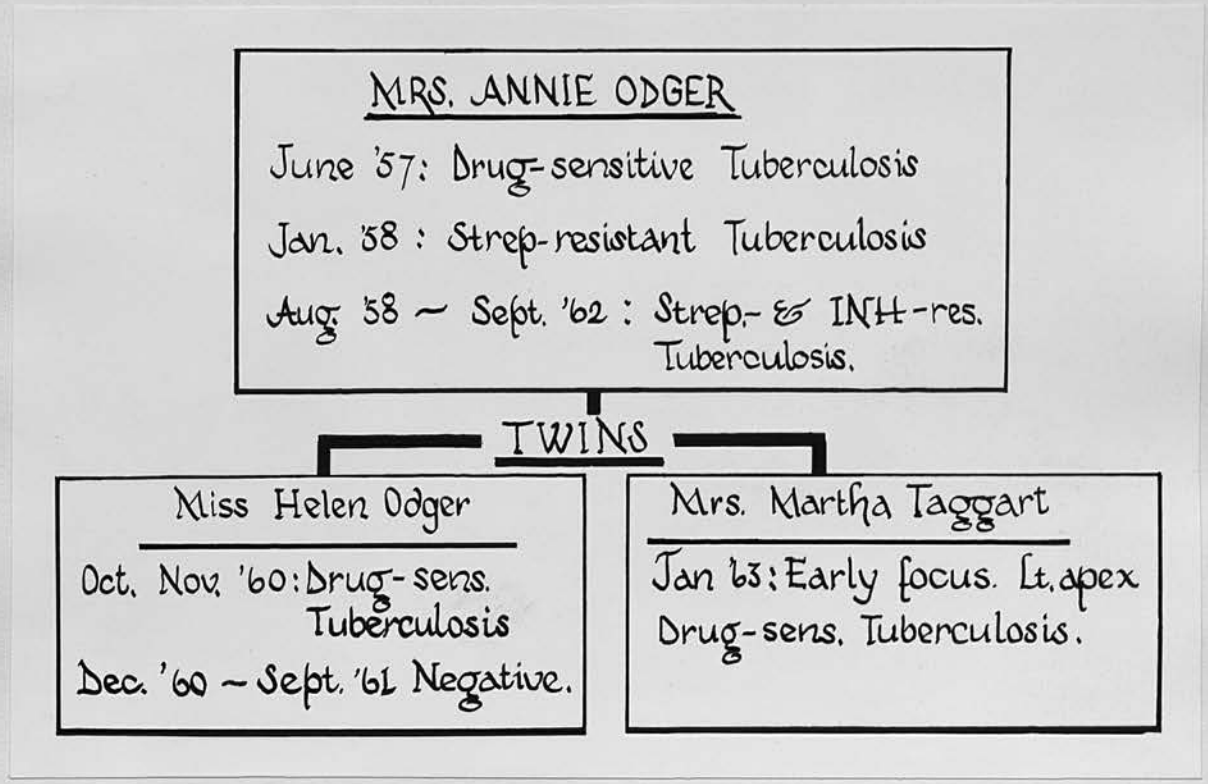


Fig: 51. Family tree of the Odgers.

Two of the daughters are twins. One, Miss H. Odger, was passed fit in the 1957 Mass X-ray Campaign; but had a cavity in the right upper lung in Oct. 1960. Happily the bacilli were fully sensitive, and six months Streptomycin, P.A.S. and I.N.H., followed by continued use of the latter two drugs, have yielded negative cultures for over two years.

The other twin, Mrs. M. Taggart, was well until 29 Jan. 1963, when haemoptysis disclosed an early lesion in the upper left lung with scanty tubercle bacilli. Her tubercle bacilli also were fully sensitive to Streptomycin, P.A.S., and I.N.H.

There would seem to be a strong presumption here that both daughters were infected by the mother who had been an open case of tuberculosis for nearly 20 years. If this is accepted cross-infection must have occurred in both girls prior to June 1958, after which the mother's bacilli were no longer of this sensitive pattern. This would give a latent period of over 4 years in the case of Mrs. Taggart, before her first early lesion was detected radiographically.

TABLE 45.

TUBERCULOSIS IN THE ODGER FAMILY

MRS. ODGER

	Streptomycin	P.A.S.	I.N.H.	Viomycin	Cycloserine
7.6.57	$\frac{1}{2}$" = Sens$^{\pm}$ 2/3	2" = Sens 0/2	1" = Sens 0/1	$\frac{3}{4}$" = Sens	
29.1.58	$\frac{1}{4}$" = Sens$^{\pm}$ 1/30	2" = Sens$^{\pm}$ 6/2	1" = Sens$^{\pm}$ 6/1	1" = Sens	
15.8.58	0" = Res. 8/30	$1\frac{1}{2}$" = Sens$^{\pm}$ 36/2 4/10	0" = Res. ∞/1 2/5	$\frac{3}{4}$" = Sens.	
4.5.60	0" = Res. 20/30	$1\frac{1}{2}$" = Sens$^{\pm}$ 15/2	0" = Res. 3/5	1" = Sens.	> 2" = Sens,
30.5.61	0" = Res. ∞/30	2" = Sens. 0/2	0" = Res. 20/5	$\frac{1}{2}$" = Sens$^{\pm}$	$\frac{3}{4}$" = Sens$^{\pm}$
21.9.62	0" = Res. ∞/30 32X = Res.	$\frac{3}{4}$" = Sens$^{\pm}$ 15/10 5/100 16X = Res.	0" = Res. ∞/1 10/5 8X = Res.	1" = Sens. 0/50 2X = Sens.	1" + 3 = Sens$^{\pm}$ 8X = Res.
22.1.64	0" = Res. ∞/30 16X = Res.	$\frac{1}{2}$" = Sens$^{\pm}$ 40/10 7/100 16X = Res.	0" = Res. ∞/1 3/5 32X= Res.	$\frac{3}{4}$"+4= Sens$^{\pm}$ ∞/50 15/100 6/250 32X= Res.	0" = Res. 8X = Res.
17.4.64	0" = Res. ∞/30	$\frac{1}{2}$" = Sens$^{\pm}$ 15/100	0" = Res. 4/5	$\frac{1}{2}$"+4= Sens$^{\pm}$ 12/100 6/250	0" = Res.

TABLE 45 (cont).

TUBERCULOSIS IN THE ODGER FAMILY.

MISS HELEN ODGER

	Streptomycin	P.A.S.	I.N.H.	Viomycin	Cycloserine
14.10.60	1"+10 = Sens± 6/30	2" = Sens± 8/2	1½" = Sens± 10/1	¾" = Sens	1¼" = Sens.

MRS. MARTHA TAGGART NEE ODGER.

6.2.63	¾" = Sens 0/3	2" = Sens. 0/2	2" = Sens 3/1	1" = Sens.	2" = Sens.
9.5.63	½" = Sens± 0/3	2" = Sens. 0/2	1¾" = Sens 0/1	¾" = Sens.	1¾" = Sens.
	4X = Sens	<1X = Sens	<1X = Sens	1X = Sens.	1X = Sens.

LEGEND:

The tests were performed in parallel on each of the dates shown, which are at 3 - 6 months' intervals. The results are quoted under each drug as follows:-

1. *The zone in inches of inhibition by Slope Diffusion Test., + mutants, if any, within zone in the case of viomycin and cycloserine tests which were kept six weeks for this. For the other drugs, read at 4 weeks, mutants were studied by:-*
2. *The mutant count on drug-incorporated slopes. To simplify the table only the critical level has been given. As a rule this has meant quoting the highes drug-concentration at which resistant mutants were seen thus:-*

"4/10" = 4 colonies on 10 μg./ml.

Sometimes, however, abrupt changes have justified quoting the concentrations at lower levels also.

The first interpretation is based on both the results of 1. and 2, and is abreviated as follows:-

Sens = sensitive, Res = resistant, Sens$^{+}_{-}$ = moderately i.e. diminishing sensitivity, Sens$^{+}_{=}$ = rapidly diminishing sensitivity.

3. Where available, that is during the last two years, the results of Resistance Ratios are given with their interpretation thus:-

"16X = Res." means 16 times as resistant as the control culture H37 Rv.

SUMMARY OF TABLE 45

It will be seen how progressive deterioration in sensitivity to all five drugs occurred in Mrs. Odger's strain over seven years, preceded in each case by the appearance of resistant mutants. This gives the first two tests used in conjunction a prognostic significance in a chronic case of this sort. No reversion to sensitivity was seen, except to a limited extent in the case of P.A.S.

This progressive deterioration was not repeated in the twin daughters, whose strains show a pattern of overall sensitivity which implies infection was transferred in the earliest years, if, as one assumes, it came from the mother. Helen's moderate degrees of resistance to three drugs suggest cross-infection could have been in early 1958, while Martha's full sensitivity must date back even earlier.

FAMILY 2. THE STUDENT BROTHERS

(See Fig: 52 and Table 46)

Graham T., a 22-year old Glasgow medical student, was found to have tuberculous cavitation of the right apex when examined after a haemoptysis in March 1961. Cultures of his tubercle bacilli were resistant to 100 μg. streptomycin and 5 μg. isoniazid, and partially to P.A.S. (10 μg. $\pm$), and two months later a further culture was unchanged. Sixteen months' treatment with cycloserine and ethionamide successfully arrested the disease giving repeatedly negative sputa.

In the course of a check on his family it was found that a brother, Robert T., in Falkirk had also had pulmonary tuberculosis, and that his cultures of tubercle bacilli in February and March 1959 were also resistant to streptomycin and isoniazid. It was interesting that he, too, had been treated, quite independently, with the same drugs - cycloserine and ethionamide - and with equal success. But still more interesting for our present investigation is the fact that Robert's sputa had yielded negative cultures for at least a year previous to Graham's first symptoms.

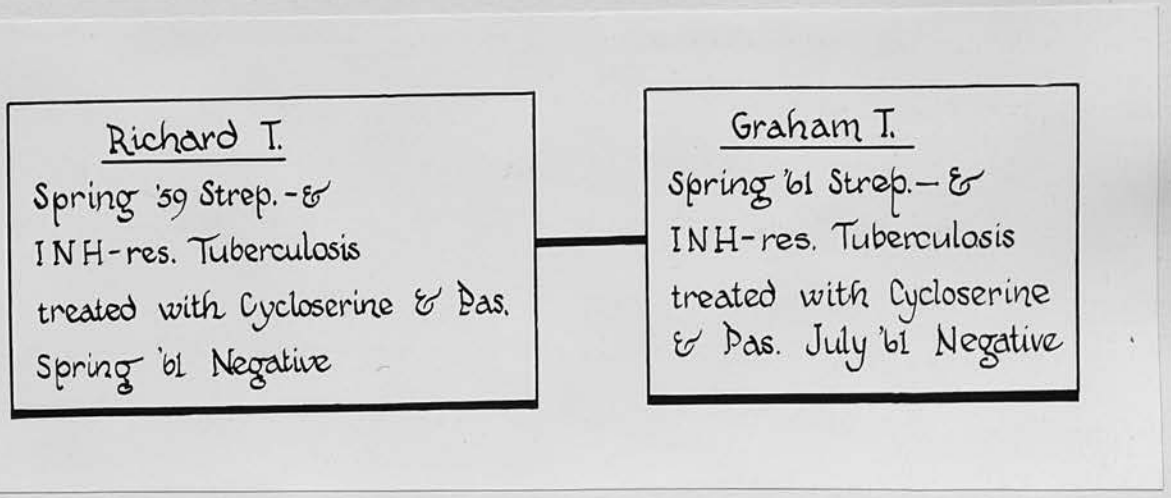


Fig: 52. The two student brothers with drug-resistant tuberculosis.

TABLE 46

SHOWING ERADICATION OF INFECTION BY CYCLOSERINE AND ETHIONAMIDE IN SPITE OF MULTIPLYING MUTANTS ALREADY RESISTANT TO STREPTOMYCIN, P.A.S. AND I.N.H. WHEN FIRST SEEN.

		Strepto-mycin	P.A.S.	I.N.H.	Viomycin	Ethion-amide	Cyclo-serine
6. 3.61	S.D.	0" = Res.	$\frac{1}{2}$" = Sens$^{\pm}$	0" = Res.	$\frac{3}{4}$" = Sens	2" = Sens	2" = Sens
	Mutants:	10/30	30/2	∞/5	-	-	-
	Drug Concn.	5/100	15/10				
			6/100				
8.5.61	S.D.	0" - Res.	$\frac{1}{2}$" = Sens$^{\pm}$	0" = Res.	$\frac{3}{4}$" = Sens	2"=Sens	$\frac{3}{4}$" = Sens
	Mutants:	40/30	20/100	∞/5	-	-	-
	Drug Conc.	5/100					
28.7.61	and since:	No growth.					

S.D. = Slope Diffusion Test, Res. = resistant, Sens = sensitive,

- = no mutants seen.

FAMILY 3. EXTINCTION OF A FAMILY BY TUBERCULOSIS

(See Fig: 53 and Table 47)

If we regard successful parasitism as the survival of a micro-organism in its host, in the next example the drug-resistant tubercle bacilli have over-reached themselves and failed. The father, mother and married daughter all died from the disease; but who was the originator was never discovered.

Mrs. Sarah Craig harboured tubercle bacilli already resistant to isoniazid and streptomycin when cultures were first tested in August 1956. Between February 1957 and July 1958 there was reversion to sensitivity to isoniazid; but in November 1958 both drugs were again ineffective and death occurred in September 1959 at Belvidere Hospital from isoniazid- and streptomycin-resistant tuberculosis.

Meanwhile in February 1958 both parents were examined and both had isoniazid-resistant but streptomycin-sensitive tuberculosis. This pattern persisted in the cultures from the father, William Laurie, and by April 1958 he was dead in Gartloch Mental Hospital. The mother, Ellen Laurie, survived three years, dying in Robroyston Sanatorium in February 1961.

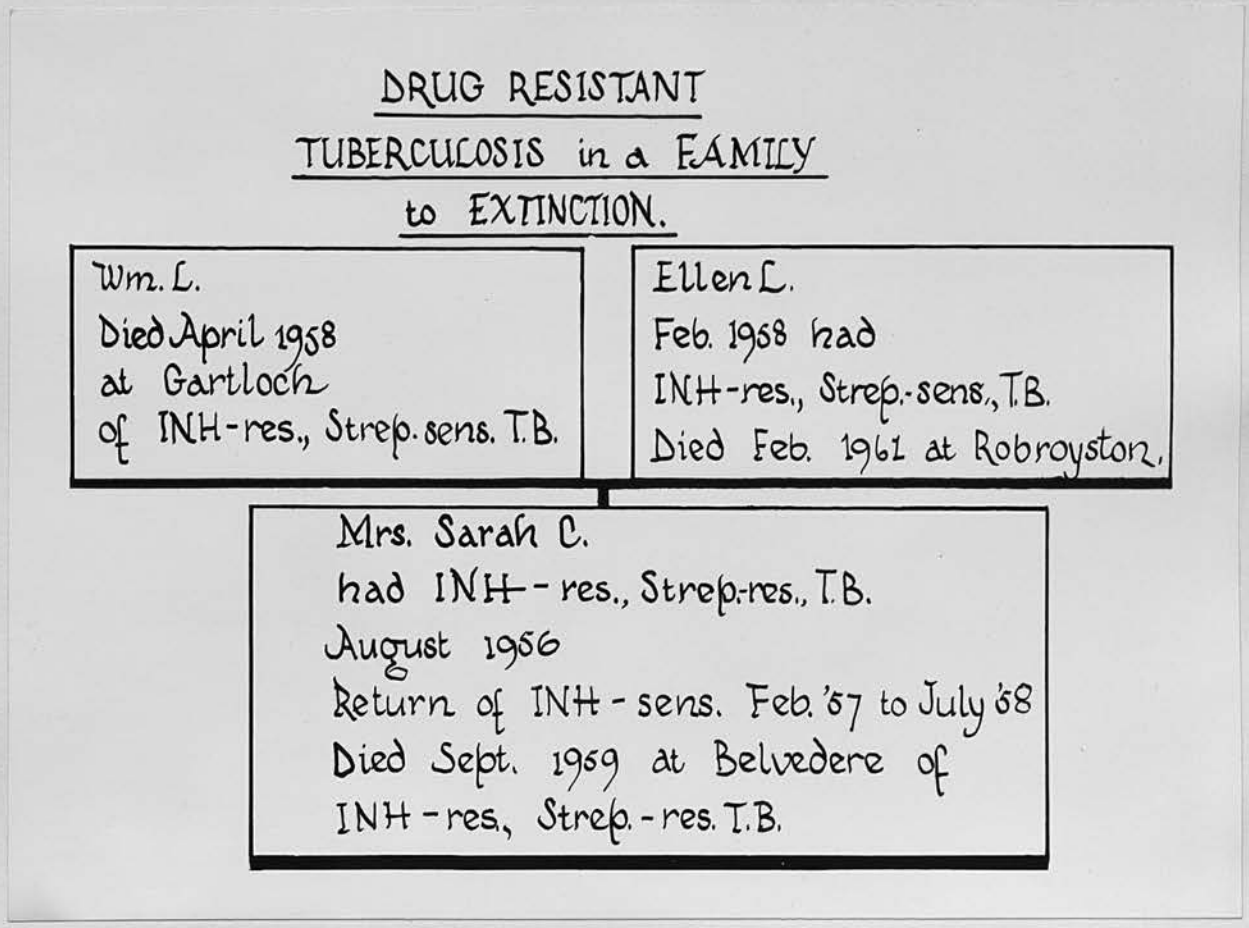


Fig: 53. Family tree of the Lauries showing their extermination by drug-resistant tuberculosis.

TABLE 47

AN EXTERMINATED FAMILY

Mrs. Sarah Craig

		Streptomycin	P.A.S.	I.N.H.	Viomycin
30. 8.56	S.D.	0" = Res.	2" = Sens	0" = Res	$1\frac{1}{4}$" = Sens
	Mutants	∞ /30	1/2	50/1	-
	Drug conc.		0/10	0/5	
22. 2.57	"	$\frac{1}{4}$" = Sens ±	2" = Sens	$\frac{3}{4}$" = Sens ±	1" = Sens
		15/30	1/2	20/1	-
		0/100		0/5	
9. 5.58	"	0" = Res	1" = Sens±	1" = Sens	$\frac{3}{4}$" = Sens
		∞/30	30/2	0/1	-
			2/10		
6.11.58	"	0" = Res	1" = Sens ±	0" = Res	1" = Sens
		50/30	12/10	∞/1	-
		20/100	6/100	12/5	

Mrs. Ellen Laurie, her mother

17. 2.58	"	1" = Sen ±	1" = Sens ±	0" = Res
		2/10	15/2	∞/1
		0/30	0/10	0/5

William Laurie, her father

17. 2.58	"	1" = Sens	1" =Sens ±	0" = Res
		0/3	1/2	30/1
				0/5

S.D. = Slope Diffusion Test, Res. = resistant, Sens = sensitive,

- = no mutants seen.

FAMILY 4. THE O'NEILS

Synopsis

Infection of a girl with tubercle bacilli with multiple drug-resistance by the mother before the latter's death (Table 48).

Mrs. Cecilia O'Neil, the mother of four children, was put on streptomycin in Nov. 1951 for tuberculous infiltration of the right lung. By Oct. 1953 both lungs were affected and by May 1954 there were cavities in the right. I.N.H. and P.A.S. were then added to the Streptomycin for some months until a hospital bed became available in Sept. 1954. The patient then continued on Streptomycin and P.A.S. until March 1955 when isoniazid was added. In March 1956 the Streptomycin was discontinued following a report that the bacilli were resistant to Streptomycin and I.N.H., sensitive to P.A.S. and viomycin. In 1957 Nupasal replaced I.N.H. (to which it is, however, only an analogue) and in 1958 Cycloserine was used instead with P.A.S. She asked for her discharge to attend her large family, and this was accepted with misgiving because of the known mental disturbance with cycloserine. Later in 1958 use of Streptomycin, P.A.S. and I.N.H. was again attempted.

By Oct. 1959 Mrs. O'Neil's tubercle bacilli were resistant to 30 μg. Streptomycin and 5 μg. I.N.H., besides

being partially resistant to P.A.S. (10μg). By March 1960 resistance to 100μg. streptomycin, 5μg. I.N.H. and 100μg. P.A.S. was reached, and during the next four months resistance to 5μg. Dipasic and 100μg. Viomycin followed. By Jan. 1961 cycloserine resistance completed the picture.

During the last months of 1960 she was being treated with ethionamide and P.A.S.; but could not tolerate the former drug. Moreover she realised only too well that she had infected her eldest daughter Sadie who was nursing her, and she threatened suicide. She was admitted to a mental hospital and all chemotherapy was stopped; but in Feb. 1961 she had a haemoptysis and died almost instantly.

The daughter, Sadie, was a weaver. X-rays had shown a healthy chest in March 1958; but cough and lassitude in the winter of 1959-1960 led to a diagnosis of active bilateral disease with right mid-zone cavitation, and the admission of mother and daughter together into hospital. Her sputum culture taken the same day as her mother's, 16.2.60, was an exact replica in drug-sensitivities, namely completely resistant to Streptomycin 30μg. and I.N.H. 5μg. with only partial sensitivity to P.A.S. In the following months resistance to dipasic and cycloserine ensued even more rapidly than in her mother's bacilli, though sensitivity to viomycin

was retained. In August and Sept. 1960 there was some reversion to sensitivity to Streptomycin and cycloserine while she was being treated with P.A.S. and kanamycin. The kanamycin brought on Monilial vulvitis, and in August 1960 viomycin was used with ethionamide and P.A.S.

Sadie took her mother's death with fortitude, and made steady progress until May 1961 when deterioration in the right lung prompted the addition of pyrazinamide to her drugs. In August 1961 we obtained our first negative culture, then a positive, then in Oct. a second negative. After two months' convalescence at Irvine the patient has spent 1962 at home on P.A.S., pyrazinamide and ethionamide and is making very slow but steady progress.

TABLE 48.

INFECTION FROM DYING MOTHER WITH TUBERCLE BACILLI SHOWING MULTIPLE DRUG-RESISTANCE

MRS. CECILIA O'NEIL

		Streptomycin	P.A.S.	I.N.H.
5.3.56	Slope Diffusion	0" = Res.	2" = Sens.	0" = Res.
	Mutants/Drug Concn:	50/30 0/100	6/2 0/10	∞/1 0/5
16.4.58	"	0" = Res. 30/30 0/100	1" = Sens± 30/2 20/10	0" = Res. ∞/1 ∞/5
9.7.58	"	0" = Res. 20/30 0/100	¾" = Sens± ∞/10 30/100	0" = Res. ∞/1 20/5
16.2.60	"	0" = Res. ∞/30 30/100	¾" = Sens± 100/10 10/100	0" = Res. ∞/5 7/100
4.7.60	"	0" = Res. ∞/30 12/100	½" = Sens± 40/10 10/100	0" = Res. ∞/1 20/5 0/100
16.1.61	"	0" = Res. ∞/30 50/100	½" = Sens± 30/10 15/100	0" = Res. 20/5 0/100

Deceased.

TABLE 48 (cont)

MISS SADIE O'NEIL

		Streptomycin	P.A.S.	I.N.H.
16.2.60	Slope Diffusion:	0" = Res	1" = Sens±	0" = Res.
	Mutants/Drug Concn:	20/30 0/100	∞/2 20/10	∞/1 0/5
5.8.60	"	0" = Res. 20/30 0/100	¾" = Sens± 50/10 15/100	0" = Res. ∞/1 ∞/5
5.12.60	"	0" = Res. 4/30	½" = Sens± 15/10	0" = Res. ∞/5
5.5.61	"	¼" = Sens± 6/30	½" = Sens± 50/2 10/10	0" = Res. ∞/5

3.7.61, 8.8.61, 7.9.61, 5.10.61, and 2.11.61. . . . No growth.

LEGEND:

Res. = resistant, Sens. = sensitive, Sens± = moderately sensitive, Sens± = only slightly sensitive. These estimates are based on both the Slope Diffusion Test's zone of inhibition and also on the count of mutant colonies on the series of slopes incorporating drugs. For clarity only the critical levels of these have been quoted. Thus "30/2 20/10" for P.A.S. means that there was a count of 30 colonies on 2 μg./ml, 20 on 10 μg./ml., and none on a higher concentration.

SUMMARY

It will be noticed that shortly before the death of the mother her daughter became an open case of tuberculosis also, and that her initial resistances to drugs matched the final ones of the mother. Resistance to I.N.H. proved irreversible in both subjects, as has been our general experience. With the other two drugs, however, there is a tendency to fluctuation back and forth in the degree of resistance, preceded in each case by a rise or fall in the mutant count. In the case of P.A.S. these never exceeded a countable number, and this is reflected in the Slope Diffusion Test, where a residual zone always remained with P.A.S. This agrees with reports suggesting that P.A.S-resistance, in fact, is never absolute.

THE ECOLOGY OF DRUG-RESISTANT TUBERCLE BACILLI

Early American reports of streptomycin-resistant tuberculosis, or its absence, may have induced a false sense of security based as they were on experience in sanatoria. Thus in 1949 Furtos and Doane [148] announced that they had treated 385 cases safely together. But only a year later Arany and Lewis [5], when describing a similar group of 368 cases treated unscathed in open wards, admitted that three of the sanatorium employees had become infected, one with

streptomycin-resistant bacilli. This suggested that while there might not be much risk of resistant organisms causing superinfections, the position was totally different with primary disease. The following case is probably an instance of this among business associates; though it remains a tantalising enigma who first infected the other.

Bernard Rinaldi, the 52-year old proprietor of a public house in South-eastern Glasgow, was admitted to hospital in June 1963 with pulmonary tuberculosis. This had been diagnosed radiologically as an extensive bilateral infection, which had caused an haemoptysis; but he admitted that for two years he had been unable to work because of "chronic bronchitis". A week later Rinalds's 61-year old barman, Lewis Bohme, was occupying the adjoining bed. He, too, had had chronic bronchitis for many years; but had showed no evidence of tuberculosis until the previous month. Moreover he had been under more or less continuous medical attention with sputum examinations and X-ray examinations, and it was following an attack of left basal pneumonia in May 1963 that he was found to have extensive cavitation on the opposite side.

Sputa from both, taken on the 19th. and 27th. June respectively, grew tubercle bacilli with the following characteristics:-

TABLE 49

INITIAL DRUG-SENSITIVITY TESTS ON TUBERCLE BACILLI FROM A PUBLICAN AND HIS BARMAN

B. Rinaldi 19.6.63 Cult: 20758

	H37 Rv μg	Test Cult. μg	Result
Streptomycin	2 : 4	32 : -	16 × Res.
P.A.S.	1 : 2	8 : -	8 × Res.
I.N.H.	0.06 : 0.125	0.25 : 0.5	4 × ? Res.
Viomycin	16 : 32	64 : 128	4 × ? Res.
Cycloserine	8 : 16	8 : 16	1 × Sens.
Ethionamide	10 : 20	10 : 20	1 × Sens.

L. Bohme 27.6.63 Cult: 21849

	H37 Rv μg	Test Cult. μg	Result
Streptomycin	2 : 4	32 : -	16 × Res.
P.A.S.	0.25 : 0.5	8 : -	32 × Res.
I.N.H.	0.3 : 0.6	1 : 50	32 × Res.
Viomycin	16 : 32	32 : 64	2 × Sens.
Cycloserine	8 : 16	8 : 16	1 × Sens.
Ethionamide	10 : 20	20 : 40	2 × Sens.

TABLE 49 (Cont.)

By Slope Diffusion + Mutant Counts on drug-incorporated media:-

B. Rinaldi *19.6.63* *Cult: 20758*

	Slope Diffusion	Mutant Counts Drug-concn.	Result
Streptomycin	$\frac{1}{4}$"	1/30	Sens.±
P.A.S.	$1\frac{1}{2}$"	20/2, 10/10, 0/100	Sens ±
I.N.H.	0"	∞/1, 0/5	Res.
Viomycin	1"	0	Sens.
Cycloserine	$1\frac{1}{2}$"	0	Sens.
Ethionamide	$1\frac{1}{2}$"	0	Sens.

L. Bohme *27.6.63* *Cult: 21849*

	Slope Diffusion	Mutant Counts Drug-concn.	Result
Streptomycin	$\frac{1}{4}$"	0	Sens.±
P.A.S.	$1\frac{1}{2}$"	30	Sens.±
I.N.H.	0"	∞	Res.
Viomycin	1"	0	Sens.
Cycloserine	$1\frac{1}{4}$"	0	Sens.
Ethionamide	$1\frac{1}{2}$"	0	Sens.

The close correspondence between the two sets of results is at once apparent; but it is particularly striking by the second method. Partly this is because a shift in the degree of sensitivity to P.A.S., and I.N.H., has occurred in the control culture H37 Rv between the tests on the two men, which were not performed together, and such slight changes, inseparable from a biological 'control' are reflected inevitably in the ratios.

Some 5-6 weeks later on 7.8.1963 both patients again yielded positive cultures, which were not tested for sensitivity. Meanwhile triple drug therapy had begun, 200 mg. isoniazid and 15 G. P.A.S. being given daily, and $\frac{3}{4}$ G. streptomycin thrice a week. After six months both men were discharged, with pyrazinamide $2\frac{1}{2}$ G. replacing the isoniazid. Rinaldi had begged to be allowed to supervise his languishing business from bed at home, and a positive sputum in January 1964 gave these results:-

TABLE 50

SUBSEQUENT DRUG-SENSITIVITY TESTS ON TUBERCLE BACILLI FROM B. RINALDI

Culture No. 433/64 *By Resistance Ratios:-* *7.1.64*

	H37 Rv	Test Culture	Results
	µg	µg	
Streptomycin	2 : 4	32 : -	16 × Res.
P.A.S.	0.5 : 1	8 : -	16 × Res.
I.N.H.	0.03 : 0.06	1 : 50	32 × Res.
Viomycin	16 : 32	32 : 64	2 × Sens.
Cycloserine	8 : 16	8 : 16	1 × Sens.
Ethionamide	20 : 40	20 : 40	1 × Sens.

and by Slope Diffusion + Mutant Counts on drug-incorporated media:-

Culture No. 433/64 *7.1.64*

	Slope Diffusion	Mutants/Drug-Conc.	Results
Streptomycin	$\frac{1}{4}$"	∞/1, 20/3, 10/10, 0/30	Res.
P.A.S.	$1\frac{1}{2}$"	50/2, 20/10, 0/100	Sens ±
I.N.H.	0"	∞/1, 0/5	Res.
Viomycin	1"	0	Sens.
Cycloserine	$1\frac{1}{4}$"	0	Sens.
Ethionamide	$1\frac{1}{2}$"	0	Sens.

It will be seen by comparing successive tests that the results for isoniazid, and viomycin (which was not used for therapy), are more consistent by the Slope Diffusion and Mutant Count method, and that this procedure also shows clearly the deterioration in the counts of resistant mutants with both streptomycin and P.A.S.

Lewis Bohme had also been discharged prematurely; because he soon lapsed on his treatment, the residual cavity enlarged, and in May 1964 he was readmitted to hospital. The more severe disease in his case, following a well-authenticated earlier history of no tuberculosis, suggests that he was the victim, and that Rinaldi was the source of his drug-resistant infection.

THE WORLDWIDE EMERGENCE OF DRUG-RESISTANT STRAINS

McDermott [277] has pointed out that in the world problem of drug-resistant tuberculosis, isoniazid and streptomycin are at present the drugs that matter, isonizid-resistance being the key problem.

Ten years ago the vast majority of tubercle bacilli were still highly sensitive to these drugs as we saw in the case of streptomycin in Table 38 ; but only five years later in 1958 the International Union Against Tuberculosis [212, 354]

concluded that drug-resistant bacilli were already a risk in 17 countries and that the average incidence was 6.5%. By June 1961 the 14th. World Health Assembly [213] announced that important as the loss of effectiveness of the drugs was to the patient, it was "not nearly so important as the spread of drug-resistant Mycobacteria in the community".

In this chapter we have seen that drug-resistant tuberculosis is virtually a new disease. Its apparent restriction to primary cases rather than to superinfection of those already phthisic suggests, at least, that there is as yet no accompanying exaltation of virulence. Slope Diffusion Tests show that it arises from mutants of the classical disease, and, if mutagenicity also increased the virulence, then indeed the small groups of cross-infections which we have described among families and associates could become potential epidemics.

PART IV.

AN EVOLUTIONARY THEORY

OF EPIDEMICS

CHAPTER 12. NATURAL SELECTION AMONG PARASITES

In the seventeenth century Sydenham [260] already looked forward to the time when his work would "be finished by posterity, and the whole series of epidemics be exhibited to view as they shall succeed each other for the future". The more credulous Sir. Thomas Browne [466] a few years earlier had written: "Some think there were few consumptions in the old world, when men lived much upon milk; and that the ancient inhabitants of this island were less troubled with coughs when they went naked and slept in caves and woods, than men now in chambers and featherbeds .... Some will allow no diseases to be new, others think that many old ones are ceased: and that such which are esteemed new, will have but their time". However irrational compared with Sydenham, his observations seem now to have been curiously prophetic, because through all the rise and fall of epidemic periodicities there are quite clearly certain long-term trends suggestive of a slow evolution.

For our studies of the rise and fall of epidemics have confirmed the remarkable fact that many such fluctuations return at most exact intervals, and that the number of these precise repetitions is far greater than could be explained by mere coincidence. This suggests that the relationships

between host and parasite, disturbance of which precipitates epidemics, must be the subject of some regularly recurring natural phenomena.

No doubt the changing seasons, and alternating events like school terms play a part; but there still remain unexplained the most conspicuous cycles - those long waves extending over years or even decades.

Pasteur [339] originally supposed that there were present in nature mixtures of virulent and attenuated organisms in varying proportions. Later, convinced that the virulence itself could vary, he realised with disquiet that here could be the reason of the spontaneous appearance of diseases such as smallpox, syphilis and plague in the past, and of the rise of the great epidemics, if only an explanation could be found for what he called the "cosmic influences" controlling these universal effects. He could only suppose [340] that a property inherent in the oxygen of the air would eventually limit epidemics, which arose by successive passages in lower animals preceding transfer to man.

We have shown in Chapters 7 and 8 that bacteria exposed to antibiotics to which they are susceptible will be inhibited except for a few resistant mutants which survive to grow on. By experiments with known numbers of bacteria

the proportion of such mutants in any population of organisms could be calculated, and such experiments showed that these mutations were occurring at a definite fixed rate, constant for any one species, and therefore predictable. As "back-mutations" can also be seen in the reverse direction, we could have here those basic phenomena of cyclical change and counter-change which are needed to explain the mysterious fluctuations of epidemic disease.

Of course there must be a host of separate distinct mutations, affecting such diverse characteristics as altered colonial morphology and virulence as well as resistance to antibiotics, occurring in all directions and to all degrees simultaneously. Probably all that our strongest drugs can do is to interfere with the back-mutations so that the to and fro equilibrium of natural mutations becomes for a time one way only. It could follow, too, that any bacterium by the mere spread by its multiplication could be a menace, because virulent mutations happening at a fixed rate will likewide be a relatively frequent occurrence. If drug-resistant and virulent organisms are alike so largely children of chance, one may well wonder whether any of the so-called harmless commensals, which abound in sputum for instance, can safely be ignored.

THE ORIGIN OF PARASITIC MICRO-ORGANISMS

Microscopic unicellular fossils have been claimed with some authority [70, 257] in meteorites, which would envisage living primaeval organisms back in unimaginable aeons of time. Burnet [50], less equivocally, has pointed out the remarkable resemblances between all living matter, in that all creatures possessing protoplasm have the same twenty-eight amino-acids constituting the proteins, although an almost infinite number are theoretically possible. This makes man and bacteria, and for that matter viruses, more alike than unlike. He further makes the point that "practically all animals live at the expense of some other organism", so that even a protozoan lives on bacteria and algae. Thus parasitism, as generally understood, is only a matter of degree.

Xalabarder and Cullell by extensive use of electron microscopy have staunchly maintained that extrusion and fusion of nuclear protoplasm regularly occur between bacteria in the lag-phase, which they therefore regard as the decisive step in the growth cycle rather than the accepted binary fission of subsequent logarithmic reproduction [491]. Such as interchange of protoplasm, if it really does occur with any regularity, would enormously facilitate mutations. But even Xalabarder admits that there is a lack of such tropism

and fusion of the two nuclei when contiguous bacteria are of different species [489].

Burnet [50] noted the significant fact that when "bacteria produce disease, the region primarily involved is almost always that which normally harbours those harmless bacteria which the pathogenic type resembles. Obvious examples are the meningococcus morphologically indistinguishable from other Neisseria in the pharynx, and the Shigellae and Salmonellae so like the other Enterobacteriaceae in the gut. In fact it seems to be almost the rule for pathogens to have their counterpart in some co-existing commensal. There is Borrelia refringens to match Borrelia vincenti and Treponema calligyrum matching Treponema pallidum. The same parallelism is seen with parasitic protozoa. Entamoeba histolytica and the harmless Entamoeba coli are a comparable pair, and here the much larger size allows some suggestive observations. In acute amoebiasis Entamoebae histolytica are big, engorged with blood cells, and rarely encysting. But in the 'carrier' state of the host smaller amoebae appear, "E. dispar or minuta" and it is said that they live on bacteria, seldom exceed 10 μ in size and are prone to encyst [198]. If the large amoebae do not in fact encyst, they must derive from the smaller ones, and it has been suggested that the transformation may well be a mutation, and that the two apparently different species are but one.

Other living creatures, such as bacteria, are necessary for the survival of E. histolytica in vitro, and it may be that bacterial infection predisposes to human amoebiosis and explains the effectiveness of anti-bacterial treatment in the acute disease [120].

Zinsser quoted by Burnet [50] has postulated that where infection is always quickly fatal to the host, as with the Rickettsiae of typhus in the louse, one must assume that the parasitism is of only recent times and that adaptation of the parasite to the host is still far from complete. By contrast some viruses, such as Herpes simplex in the human mouth, have evolved such a perfect equilibrium that they are tolerated throughout the normal lifetime of the host. Examples could be found of all degrees between these extremes, with the viruses on the whole better adapted to parasitism than the bacteria. Perhaps the pathogenic Neisseria represent a transitional stage. Their characteristic presence in the cytoplasm of polymorph cells is generally ascribed to phagocytosis prior to destruction; but both they and the white blood cell often look healthy enough, and the profusion of cocci in one polymorph when others round it are unoccupied may imply the first steps in intracellular parasitism.

DISCONTINUOUS CHANGE

The doctrine of monomorphism, that bacteria multiplying by binary fission stay true to type in shape, growth and functions, dates back to the time of Koch. Within a decade Eisenberg had shown such things as involution forms [177, 178] and in 1906 Neisser [326] suggested that the abnormal shapes of E. coli mutabile were due to mutation. Most of the variationists, those who accepted this view, concluded that such mutations as the change from smooth to rough in bacterial colonies were transient departures from the normal; but a few, the cyclical variationists, detected a certain purposiveness in these modifying cultures, and suggested that such serial transformations must lead in a certain direction.

If we agree with Burnet [50] that "growth, reproduction and mutation are the fundamental characteristics of life," we must accept that evolution, the outcome of successive mutations is inevitable from life itself. Sexual reproduction in higher animals allows continuous variation so that no two individuals are quite alike, and on the wide choice which this offers will ultimately depend survival in an ever-changing world. Among bacteria the genetic unions, whether by lysogenic conversion, transformation and transduction as described in Chapter 5, or by the direct cell fusion and

interchange of Xalaborder quoted above, must only seldom occur. Even spontaneous mutations and the selection of successful variants is relatively rare; but, because of the huge number of individuals concerned, evolution is achieved, and hence bacteria are remarkable for their plasticity and apparent ease of adaptation.

Evolution depends on successive mutations, and is fortuitous in so far as these are chance occurrences. An example of evolution in our own day is the loss of scent this century by musk. Epidemics represent these successful mutations in a parasite, giving increased virulence etc., and if they continue to succeed they will establish as a new type and hence a distinct new species may evolve.

Possible examples of this "discontinuity" in historical times are mumps orchitis changing to primarily parotitis, infantile paralysis changing to adult polio-encephalitis, the rise of herpes zoster rather than chickenpox, and perhaps syphilis from syphiloids. Many pathogens have commensal counterparts e.g. Shigellae and the Paracolon bacilli, Anthrax Bacilli and the anthracoids, Meningococcus and N. catarrhalis, Borrelia vincenti and refringens, Diphtheria Bacilli and the diphtheroids.

THE EVOLUTION OF A NEW BACTERIAL SPECIES

Until 1946 bacteria, living an apparently asexual existence with vegetative multiplication, were regarded as lying outside the main stream of biological evolution. In that year Lederberg and Tatum [262] working with E. coli showed that two biochemically distinct strains when mixed and cultured together produced progeny of the order of one in a million possessing the characters of both. More recently actual sexual differentiation has been shown in E. coli, and the male-determining gene "F" has even been transferred to other species so that E. coli, Salmonellae and Shigellae have been crossed with each other as well as among themselves[216]. It seems reasonable to suppose that some bacteria must possess in their protoplasm sexual differentiation with all its genetic consequences fundamentally comparable to that in higher creatures.

Ravin [360] has pointed out that although mutations occur at random "the living universe is not a vast community of genetically interacting organisms". For if it were the whole merit of achieving adaptation by selection would be lost again as the better variants would be just as fugitive as the more poorly adapted progeny among the maze of further mutations. In higher creatures there are "isolating mechanisms" which limit breeding for practical purposes to the same species.

Flowers bloom in their proper season, animals mate only with their like, and so on. Similar isolating mechanisms must occur among bacteria to limit the randomness of their mutations, and they have been shown to exist. Excessive conjugations are limited by the incompatibility of very unlike pairs, transductions are segregated by the specificity of the phage responsible, and rejection of incompatible D.N.A. from an unsuitable partner would prevent transformations which were too bizarre.

The isolating mechanism which limits mutants from an infinite variety to a finite number of species has been shown to be itself induced by mutation and recombination. If two differing bacterial populations from a common stock continue side by side they may well remain just a heterogeneous population. But if each is adjusting to a distinct ecological niche, the isolating mechanism will ensure that they continue to be differentiated. If it is advantageous to evolution the isolating mechanism, and the mutations which it controls, will persist and must ultimately create a distinct new species. We have here an example of 'cybernetics' in that we are studying an automatically self-regulating system [338, 465].

RHYTHMIC BEHAVIOUR IN LIVING CREATURES

Professor Cloudsley-Thompson [72], after ten years' study of the clock-like regularity of phenomena in crustaces and other arthropods, concluded that rhythmic phenomena permeate all aspects of life. Among parasites especially periodicity is remarkably precise [430]. The recurring bouts of fever in malaria may occur on alternate or every third day according to the life-cycle of the plasmodium responsible; but the acme of the fever when there is the maximum disintegration of infected blood cells always occurs between 6 and 8 a.m. in a day worker. A change of working hours by the host is promptly reflected in a change of rhythm by the parasite. Why this should be so, or indeed how all the plasmodia keep in step anyway, is a complete mystery.

In Asiatic filariasis the maximum migration of microfilariae from the lungs to the peripheral blood takes place around midnight, while in African loa loa the timing is noon. Probably these rhythms are linked to the life cycles of the associated insect vectors. Female mosquitoes needing blood to mature their ova bite by night or around dawn, while the vector of loa loa is the mango fly biting in the heat of the day. But thisstill does not explain the timing mechanism within the parasite. Possibly the drop in oxygen in the blood during sleep is detected, or minor temperature changes, or the rise in waste products; but this

is but speculation, one can only marvel at what Cloudsley-Thompson terms "biological clocks" in nature. Intermittent emergence of mutations implies a similar periodicity, and this thesis shows how their rhythmical recurrences are not only possible but are in fact inevitable.

THE BALANCE OF NATURE

Recent work [40, 262, 475] suggests that recombination among bacteria depends in the first instance on chance collisions between comparable organisms, and only subsequently on all the highly specific genetic transfers which we have described. In such randomness preceding specificity there might be thought to be an analogy with the very specific natural selection which succeeds supposedly chance mutations.

But are any living organisms in reality creatures of chance? Equilibrium between existing species is a natural tendency of the world in which we live, and this must imply, in the case of a parasite and its host, a constant mobilis in mobile [393]. Disturbance of this equilibrium could result from a major change in one partner or lesser changes in both. The resulting imbalance will be "disease" in that host, and if it persists as a gross imbalance favouring the parasite an "epidemic" may result. Permanent successful change will mean evolution towards a new species as envisaged by Darwin.

The Lederbergs [261] postulated that bacteria adapt to new environments by "spontaneous mutation and population selection". If, by spontaneity is meant unrestrained freedom, our experiments with E. coli and Myco. tuberculosis show, on the contrary, that bacterial mutations are highly ordered in number and frequency. If the mutation rates of bacteria are fixed in time so must be the appearance of their dependant characters, and rising at regular intervals like beats will be major phenomena like drug-resistance, virulence and epidemicity, in fact the pulsing progress of evolution itself.

ACKNOWLEDGMENTS

I should like to thank Dr. J. C. J. Ives for advice and encouragement, and Drs. R. S. Kennedy and D. Macfarlane who were in charge of the tuberculous cases. Dr. B. A. Woodger and Dr. Katherine M. Miller sent me cultures of Anonymous Mycobacteria and Dr. L. G. Bruce generously helped with parallel Resistance Ratio Tests.

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